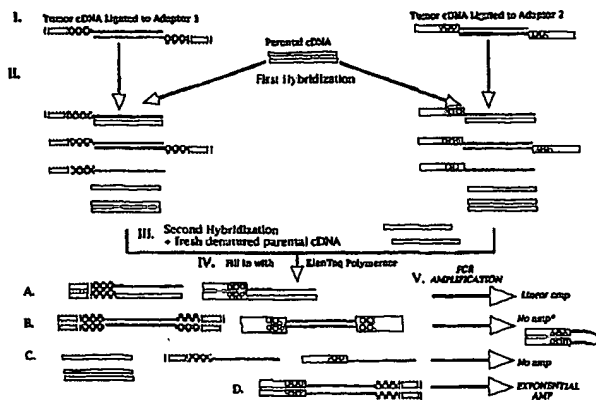


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<b>(21) International Application Number:</b> <b>PCT/US98/24029</b> <b>(22) International Filing Date:</b> <b>10 November 1998 (10.11.98)</b> <b>(71) Applicant:</b> <b>UNIVERSITY OF ROCHESTER [US/US];</b> Office of Technology Transfer, 518 Hylan Building, Rochester, NY 14627 (US). <b>(72) Inventor:</b> <b>ZAUDERER, Maurice;</b> 44 Woodland Road, Pittsford, NY 14534 (US). <b>(74) Agent:</b> <b>STEFFE, Eric, K.; Sterne, Kessler, Goldstein &amp; Fox P.L.L.C.,</b> Suite 600, 1100 New York Avenue, Washington, DC 20005-3934 (US).		<b>(81) Designated States:</b> <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</b>  <b>Published</b> <i>With international search report.</i>

**(54) Title: T CELLS SPECIFIC FOR TARGET ANTIGENS AND METHODS AND VACCINES BASED THEREON****(57) Abstract**

The present invention relates to novel methods for the identification of antigens recognized by cytotoxic T cells (CTLs) and specific for human tumors, cancers, and infected cells, and the use of such antigens in immunogenic compositions or vaccines to induce regression of tumors, cancers, or infections in mammals, including humans. The invention encompasses methods for induction and isolation of cytotoxic T cells specific for human tumors, cancers and infected cells, and for improved selection of genes that encode the target antigens recognized by these specific T cells. The invention also relates to differential display methods that improve resolution of, and that reduce the frequency of false positives of DNA fragments that are differentially expressed in tumorous, cancerous, or infected tissues versus normal tissues. The invention further relates to the engineering of recombinant viruses as expression vectors for tumor, cancer, or infected cell-specific antigens.

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**Description**

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**T CELLS SPECIFIC FOR TARGET ANTIGENS  
AND METHODS AND VACCINES BASED THEREON**

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**1. INTRODUCTION**

The present invention relates to novel methods for the identification of antigens recognized by cytotoxic T cells (CTLs) and specific for human tumors, cancers, and infected cells, and the use of such antigens in immunogenic compositions or vaccines to induce regression of tumors, cancers, or infections in mammals, including humans. The invention encompasses methods for induction and isolation of cytotoxic T cells specific for human tumors, cancers or infected cells, and for improved selection of genes that encode the target antigens recognized by these specific T cells. The invention also relates to differential display methods that improve resolution of, and that reduce the frequency of false positives of DNA fragments that are differentially expressed in tumorous, cancerous, or infected tissues versus normal tissues. The invention further relates to the engineering of recombinant viruses as expression vectors for tumor, cancer, or infected cell-specific antigens.

**2. BACKGROUND OF THE INVENTION**

Current therapies for cancer include surgery, chemotherapy and radiation. The development and use of immunotherapeutic approaches, e.g., tumor targeting using antibody conjugates, "cancer vaccines", etc. is an attractive alternative, but has, to date, met with limited success for a number of reasons. The development of monoclonal antibodies specific for tumor antigens, for example, has proved difficult, in part, because antigens

5 that are recognized by monoclonal antibodies and that are  
expressed by tumors and cancer cells are often also  
expressed by normal, non-cancerous cells. In addition, the  
10 expression of membrane antigens targeted by antibodies is  
frequently modulated to permit growth of tumor variants  
5 that do not express those antigens at the cell surface. A  
cell-mediated immune response may be more effective for  
eradication of tumors both because of the different array  
15 of effector functions that participate in such responses,  
and because T cell-mediated responses target not only  
membrane antigens but any tumor-specific intracellular  
10 protein that can be processed and presented in association  
with major histocompatibility molecules. It is, for this  
20 reason, much more difficult for a tumor to evade T cell-  
surveillance by modulating membrane expression.

Immunotherapeutic approaches based on cell-  
25 mediated immune responses are likely to be more effective,  
15 but antigens that are expressed by tumors and recognized in  
cell-mediated immune responses are difficult to identify  
and to produce. Development of an effective treatment for  
30 cancer through vaccination and subsequent stimulation of  
cell-mediated immunity, has remained elusive; the  
identification of effective antigens to stimulate cell-  
20 mediated responses has been successful only in special  
cases, such as melanoma. In melanoma, the cytotoxic T cells  
35 (CTLs) that mediate a cellular immune response against  
melanoma infiltrate the tumor itself, and such CTLs can be  
harvested from the tumor and used to screen for reactivity  
40 against other melanoma tumors. Isolation of tumor  
25 infiltrating lymphocytes has, however, not been a  
successful strategy to recover cytotoxic T cells specific  
for most other tumors, in particular the epithelial cell  
45 carcinomas that give rise to greater than 80% of human  
cancer.

To address the problem of identifying effective  
30 antigens for use in vaccination, most previous work has  
50 focused on screening expression libraries with tumor-

5 specific CTLs to identify potential tumor antigens. There  
are significant limitations to the existing methods of  
identifying effective antigens, including the excessively  
laborious and inefficient screening process and the  
10 considerable difficulty in isolating tumor-specific CTLs  
5 for most types of tumors.

## 2.1. CANCER VACCINES

15 The possibility that altered features of a tumor  
cell are recognized by the immune system as non-self and  
may induce protective immunity is the basis for attempts to  
10 develop cancer vaccines. Whether or not this is a viable  
20 strategy depends on how the features of a transformed cell  
are altered. Appreciation of the central role of mutation  
in tumor transformation gave rise to the hypothesis that  
tumor antigens arise as a result of random mutation in  
25 genetically unstable cells. Although random mutations  
15 might prove immunogenic, it would be predicted that these  
would induce specific immunity unique for each tumor. This  
would be unfavorable for development of broadly effective  
30 tumor vaccines. An alternate hypothesis, however, is that  
a tumor antigen may arise as a result of systematic and  
reproducible tissue specific gene deregulation that is  
20 associated with the transformation process. This could  
35 give rise to qualitatively or quantitatively different  
expression of shared antigens in certain types of tumors  
that might be suitable targets for immunotherapy. Early  
results, demonstrating that the immunogenicity of some  
40 experimental tumors could be traced to random mutations (De  
25 Plaen, et al., 1988, Proc. Natl. Acad. Sci. USA 85:  
2274-2278; Srivastava, & Old, 1989, Immunol. Today 9: 78),  
clearly supported the first hypothesis. There is, however,  
45 no a priori reason why random mutation and systematic gene  
deregulation could not both give rise to new immunogenic  
expression in tumors. Indeed, more recent studies in both  
30 experimental tumors (Sahasrabudhe, et al., 1993, J.

5 Immunology 151:6202-6310; Torigoe, et al., 1991, J.  
Immunol. 147:3251) and human melanoma (van Der Bruggen, et  
al., 1991, Science 254:1643-1647; Brichard, et al., 1993,  
10 J. Exp. Med. 178:489-495; Kawakami, et al., 1994, Proc.  
Natl. Acad. Sci. USA 91:3515-3519; Boel, et al., 1995,  
5 Immunity 2:167-175; Van den Eynde, et al., 1995, J. Exp.  
Med. 182: 689-698) have clearly demonstrated expression of  
shared tumor antigens encoded by deregulated normal genes.  
15 The identification of MAGE-1 and other antigens common to  
different human melanoma holds great promise for the future  
development of multiple tumor vaccines.

10 In spite of the progress in melanoma, shared  
20 antigens recognized by cytotoxic T cells have not been  
described for other human tumors. The major challenge is  
technological. The most widespread and to date most  
25 successful approach to identify immunogenic molecules  
uniquely expressed in tumor cells is to screen a cDNA  
15 library with tumor-specific CTLs (cytotoxic T lymphocytes).  
Application of this strategy has led to identification of  
several gene families expressed predominantly in human  
30 melanoma. Two major limitations of this approach, however,  
are that (1) screening requires labor intensive  
transfection of numerous small pools of recombinant DNA  
20 into separate target populations in order to assay T cell  
35 stimulation by a minor component of some pool; and (2) with  
the possible exception of renal cell carcinoma,  
tumor-specific CTLs have been very difficult to isolate  
from either tumor infiltrating lymphocytes (TIL) or PBL of  
40 patients with other types of tumors, especially the  
25 epithelial cell carcinomas that comprise greater than 80%  
of human tumors. It appears that there may be tissue  
specific properties that result in tumor-specific CTLs  
being sequestered in melanoma.

45 Direct immunization with tumor-specific gene  
products may be essential to elicit an immune response  
30 against some shared tumor antigens. It has been argued  
50 that, if a tumor expressed strong antigens, it should have

5 been eradicated prior to clinical manifestation. Perhaps  
then, tumors express only weak antigens. Immunologists  
have long been interested in the issue of what makes an  
10 antigen weak or strong. There have been two major  
hypotheses. Weak antigens may be poorly processed and fail  
5 to be presented effectively to T cells. Alternatively, the  
number of T cells in the organism with appropriate  
specificity might be inadequate for a vigorous response (a  
15 so-called "hole in the repertoire"). Elucidation of the  
complex cellular process whereby antigenic peptides  
associate with MHC molecules for transport to the cell  
10 surface and presentation to T cells has been one of the  
triumphs of modern immunology. These experiments have  
20 clearly established that failure of presentation due to  
processing defects or competition from other peptides could  
render a particular peptide less immunogenic. In contrast,  
25 it has, for technical reasons, been more difficult to  
15 establish that the frequency of clonal representation in  
the T cell repertoire is an important mechanism of low  
responsiveness. Recent studies demonstrating that the  
30 relationship between immunodominant and cryptic peptides of  
a protein antigen change in T cell receptor transgenic mice  
suggest, however, that the relative frequency of  
20 peptide-specific T cells can, indeed, be a determining  
35 factor in whether a particular peptide is cryptic or  
dominant in a T cell response. This has encouraging  
implications for development of vaccines. With present day  
40 methods, it would be a complex and difficult undertaking to  
modify the way in which antigenic peptides of a tumor are  
25 processed and presented to T cells. The relative frequency  
of a specific T cell population can, however, be directly  
and effectively increased by prior vaccination. This  
45 could, therefore, be the key manipulation required to  
render an otherwise cryptic response immunoprotective.

Another major concern for the development of  
30 broadly effective human vaccines is the extreme  
polymorphism of HLA class I molecules. Class I



5 MHC:cellular peptide complexes are the target antigens for  
specific CD8+ CTLs. The cellular peptides, derived by  
degradation of endogenously synthesized proteins, are  
10 translocated into a pre-Golgi compartment where they bind  
to class I MHC molecules for transport to the cell surface.

5 The CD8 molecule contributes to the avidity of the  
interaction between T cell and target by binding to the  $\alpha 3$   
domain of the class I heavy chain. Since all endogenous  
15 proteins turn over, peptides derived from any cytoplasmic  
or nuclear protein may bind to an MHC molecule and be  
transported for presentation at the cell surface. This  
10 allows T cells to survey a much larger representation of  
cellular proteins than antibodies which are restricted to  
20 recognize conformational determinants of only those  
proteins that are either secreted or integrated at the cell  
membrane.

25 The T cell receptor antigen binding site  
15 interacts with determinants of both the peptide and the  
surrounding MHC. T cell specificity must, therefore, be  
defined in terms of an MHC:peptide complex. The  
30 specificity of peptide binding to MHC molecules is very  
broad and of relatively low affinity in comparison to the  
antigen binding sites of specific antibodies. Class  
20 I-bound peptides are generally 8-10 residues in length and  
accommodate amino acid side chains of restricted diversity  
35 at certain key positions that match pockets in the MHC  
peptide binding site. These key features of peptides that  
bind to a particular MHC molecule constitute a peptide  
40 binding motif.

25 Hence, there exists a need for methods to  
facilitate the induction and isolation of T cells specific  
for human tumors, cancers and infected cells and for  
45 methods to efficiently select the genes that encode the  
major target antigens recognized by these T cells in the  
proper MHC-context.

30

## 2.2. VACCINIA VECTORS

Poxvirus vectors are used extensively as expression vehicles for protein and antigen, e.g. vaccine antigen, expression in eukaryotic cells. Their ease of cloning and propagation in a variety of host cells has led, in particular, to the widespread use of poxvirus vectors for expression of foreign protein and as delivery vehicles for vaccine antigens (Moss, B. 1991, Science 252:1662-7).

Customarily, the foreign DNA is introduced into the poxvirus genome by homologous recombination. The target protein coding sequence is cloned behind a vaccinia promoter flanked by sequences homologous to a non-essential region in the poxvirus and the plasmid intermediate is recombined into the viral genome by homologous recombination. This methodology works efficiently for relatively small inserts tolerated by prokaryotic hosts. The method becomes less viable in cases requiring large inserts as the frequency of homologous recombination is low and decreases with increasing insert size; in cases requiring construction of labor intensive plasmid intermediates such as in expression library production; and, in cases where the propagation of DNA is not tolerated in bacteria. Hence, there is a need for improved methods of introducing large inserts at high frequency, that do not require such labor intensive genetic engineering.

Alternative methods using direct ligation vectors have been developed to efficiently construct chimeric genomes in situations not readily amenable for homologous recombination (Merchlinsky, M. et al., 1992, Virology 190:522-526; Scheifflinger, F. et al., 1992, Proc. Natl. Acad. Sci. USA. 89:9977-9981). These direct ligation protocols have obviated the need for homologous recombination to generate poxvirus chimeric genomes. In such protocols, the DNA from the genome was digested, ligated to insert DNA in vitro, and transfected into cells infected with a helper virus (Merchlinsky, M. et al., 1992,

5 Virology 190:522-526, Scheifflinger, F. et al., 1992, Proc.  
Natl. Acad. Sci. USA 89:9977-9981). In one protocol, the  
genome was digested at the unique NotI site and a DNA  
10 insert containing elements for selection or detection of  
the chimeric genomes was ligated to the genomic arms  
5 (Scheifflinger, F. et al., 1992, Proc. Natl. Acad. Sci. USA.  
89:9977-9981). This direct ligation method was described  
for the insertion of foreign DNA into the vaccinia virus  
15 genome (Pfleiderer et al., 1995, J. General Virology  
76:2957-2962). Alternatively, the vaccinia WR genome was  
modified by removing the NotI site in the HindIII F  
10 fragment and reintroducing a NotI site proximal to the  
thymidine kinase gene such that insertion of a sequence at  
20 this locus disrupts the thymidine kinase gene, allowing  
isolation of chimeric genomes via use of drug selection  
(Merchlsinsky, M. et al., 1992, Virology 190:522-526).  
25 The direct ligation vector, vNotI/tk allowed one  
15 to efficiently clone and propagate DNA inserts at least 26  
kilobase pairs in length (Merchlsinsky, M. et al., 1992,  
Virology, 190:522-526). Although, large DNA fragments were  
30 efficiently cloned into the genome, proteins encoded by the  
DNA insert will only be expressed at the low level  
corresponding to the thymidine kinase gene, a relatively  
20 weakly expressed early class gene in vaccinia. In addition,  
35 the DNA will be inserted in both orientations at the NotI  
site. Hence, there is a need for more efficient methods of  
cloning large DNA fragments into the viral genome with  
40 accompanying high levels of expression of the protein  
product encoded by the DNA insert. There also exists a  
25 need for improved direct ligation vectors. Such vectors  
will be more universally useful for the development of  
cancer vaccines.

### 3. SUMMARY OF THE INVENTION

The invention relates to methods for the identification of target antigens recognized by CTLs, and the formulation and use of such antigens in immunogenic compositions or vaccines to induce cell-mediated immunity against target cells, such as tumor cells, that express the target antigens.

Two basic approaches are described for the identification of target antigens. In one approach, CTLs generated against authentic target cells, such as tumor cells, in animals tolerized to non-target (e.g., non-tumorigenic) cellular counterparts are used to screen expression libraries made from target cell-derived (e.g., tumor-derived) DNA, RNA or cDNA to identify clones expressing target antigens. The CTLs generated by the methods described herein are not cross-reactive with normal cells, and thus are better tools for screening. Improved expression libraries are also described.

In a second approach for identifying target antigens, products of genes differentially expressed in target cells, such as tumor cells, are used to immunize animals to generate HLA-restricted CTLs which are evaluated for activity against authentic target cells. Like the first approach, this second strategy could also be particularly useful for identifying epitopes for many human tumor types where it has not been possible to generate tumor-specific CTLs directly from patients. In addition, it may identify cryptic antigens of the intact tumor cell - i.e., tumor cell products which can become immunogenic, if the representation of tumor-specific CTLs is first augmented by vaccination with that tumor cell product. Modified methods for differential display that improve resolution and reduce false positives are described.

In accordance with the present invention, the target cell is a cell to which it is desirable to induce a cell-mediated immune response. Examples of target cells in

5 the body include, but are not limited to, tumor cells,  
malignant cells, transformed cells, cells infected with a  
virus, fungus, or mycobacteria, or cells subject to any  
10 other disease condition which leads to the production of  
target antigens.

5 The invention also encompasses the high yield  
expression of candidate target antigens, and production of  
recombinant viruses for vaccine formulation.

### 3.1. ABBREVIATIONS

10 CTLs - cytotoxic T lymphocytes (T cells)  
PBL - peripheral blood lymphocytes  
20 RDA - Representational Difference Analysis  
TIL - tumor infiltrating lymphocytes

### 4. DESCRIPTION OF THE FIGURES

25 **Figure 1.** Nucleotide Sequence of p7.5/tk and pEL/tk. The  
15 nucleotide sequence of the promoter and beginning of the  
thymidine kinase gene for v7.5/tk and vEL/tk.

30 **Figure 2.** Modifications in the nucleotide sequence of the  
p7.5/tk vaccinia transfer plasmid. Four new vectors,  
20 p7.5/ATG0/tk, p7.5/ATG1/tk, p7.5/ATG3/tk, and p7.5/ATG4/tk  
have been derived as described in the text from the p7.5/tk  
35 vaccinia transfer plasmid. Each vector includes unique  
BamHI, SmaI, PstI, and SalI sites for cloning DNA inserts  
that employ either their own endogenous translation  
initiation site (in vector p7.5/ATG0/tk) or make use of a  
40 vector translation initiation site in any one of the three  
25 possible reading frames (p7.5/ATG1/tk, p7.5/ATG3/tk, and  
p7.5/ATG4/tk).

45 **Figure 3.** Schematic of the Clontech PCR SELECT™ method of  
Representational Difference Analysis. Adapted from  
30 information provided by the manufacturer.

5 Figure 4. BCA 39 tumor DNA fragments amplified by PCR  
following RDA subtraction of B/c.N parental sequences.  
Nested primers incorporated into the RDA adapters ligated  
10 to BCA 39 tumor cell cDNA were employed for sequential PCR  
amplification of the DNA fragments recovered from RDA.  
5 Bands are resolved on a 2% Metaphor agarose gel. One  
additional low molecular weight band ran off the  
illustrated gel and was recovered from a shorter  
15 electrophoretic run.

20 Figure 5. Hybridization of an RDA fragment of (A) an IAP  
pol gene or (B) a fragment of the ubiquitously expressed  
murine G3PDH cDNA to Northern blots of BCA 39 tumor RNA.  
15 micrograms of total RNA was transferred from 1% alkaline  
agarose gel to Genescreen nylon membrane by capillary blot  
25 in 10X SSC. The Northern blot was first hybridized to the  
15 <sup>32</sup>P labeled RDA clone 1 DNA (10<sup>5</sup> cpm/ml Stark's hybridization  
buffer), then stripped and hybridized with a 350 bp  
fragment of G3PDH cDNA.

30 Figure 6. RDA clones encoding fragments of IAP gene  
elements compared with the full length IAP clone MIA14.  
20 One or both terminal regions (filled rectangular box) of  
each RDA clone were sequenced to identify homology to  
35 subregions of an IAP element. The extent of overlap was  
estimated from either the fragment size or, where the  
sequence at both termini of a fragment was determined, the  
known IAP MIA14 sequence spanning the two termini of that  
40 25 fragment. In one case, clone 2.19, the two measures were  
not consistent suggesting a deletion in this IAP fragment  
in BCA 39 tumor cells.

45 Figure 7. Modified Differential Display of cDNA of  
parental cell B/c.N and tumors BCA 39, BCA 34, BCA 22, and  
30 BCB 13. Fragments of parental and tumor cell cDNA were  
50 amplified with one pair of arbitrary decamers, MR\_1 (TAC

5 AAC GAG G) and MR\_5 (GGA CCA AGT C). For each cell line,  
first strand cDNA synthesis was separately primed with MR-1  
or MR\_5. The two cDNA preparations were then pooled for  
10 PCR amplification with both MR\_1 and MR\_5. A number of  
bands can be identified that are associated with all four  
5 tumors but not with the immortalized, non-tumorigenic  
parental cell line.

15 **Figure 8.** Differential expression in tumor lines of  
differential display clone 90. Rnase protection assay: 300  
picograms of clone 90 antisense probe was hybridized with 5  
10 micrograms total RNA prior to RNase digestion and analysis  
of protected fragments on 5% denaturing PAGE.

20 **Figure 9.** Gene isolation in solution. Schematic of a  
method for selection of longer length cDNA from single  
strand circles rescued from a phagemid library. DNA  
15 fragments identified through RDA or Modified Differential  
Display are employed to select more full length cDNA.

30 **Figure 10.** Restriction Enzyme Analysis of Virus Genomes  
Using CHEF Gel. BSC-1 cells were infected at high  
20 multiplicity of infection (moi) by vaccinia WR, vEL/tk,  
v7.5/tk, or vNotI/tk. After 24 hours the cells were  
35 harvested and formed into agarose plugs. The plugs were  
equilibrated in the appropriate restriction enzyme buffer  
and 1mM PMSF for 16 hours at room temperature, incubated  
with restriction enzyme buffer, 100ng/ml Bovine Serum  
40 Albumin and 50 units NotI or ApaI for two hours at 37°C  
25 (NotI) or room temperature (ApaI) and electrophoresed in a  
1.0% agarose gel on a Bio-Rad CHEFII apparatus for 15 hours  
at 6 V/cm with a switching time of 15 seconds. The  
45 leftmost sample contains lambda DNA, the second sample  
contains undigested vaccinia DNA, and the remainder of the  
30 samples contain the DNA samples described above each well  
digested with ApaI or NotI where vEL refers to vEL/tk and  
50

5 v7.5 refers to v7.5/tk. The lower portion of the figure is a schematic map showing the location of the NotI and ApaI sites in each virus.

10 **Figure 11. Southern Blot Analysis of Viral Genomes p7.5/tk**

5 and pEL/tk. The viruses v7.5/tk and vEL/tk were used to infect a well of a 6 well dish of BSC-1 cells at high multiplicity of infection (moi) and after 48 hours the cells were harvested and the DNA was isolated using DNazol (Gibco). The final DNA product was resuspended in 50 microliters of TE 8.0 and 2.5 microliters were digested with HindIII, HindIII and ApaI, or HindIII and NotI, electrophoresed through a 1.0% agarose gel, and transferred to Nytran (Schleicher and Schuell) using a Turboblotter (Schleicher and Schuell). The samples were probed with p7.5/tk (Figure 11a) or pEL/tk (Figure 11b) labeled with <sup>32</sup>P using Random Primer DNA Labeling Kit (Bio-Rad) in QuickHyb (Stratagene). The lower portion of the figure denotes a map of the HindIII J fragment with the positions of the HindIII, NotI, and ApaI sites illustrated. The leftmost 0.5 kilobase fragment has electrophoresed off the bottom of the gel.

20 **Figure 12. Analysis of v7.5/tk and vEL/tk by PCR.** One well of a 6 well dish of BSC-1 cells was infected with v7.5/tk, vEL/tk, vNotI/tk, vpNotI, vNotI/lacZ/tk, or wild type vaccinia WR at high multiplicity of infection (moi) and after 48 hours the cells were harvested, and the DNA was isolated using DNazol (Gibco). The final DNA product was resuspended in 50 microliters of TE (10mM TrisHCl, pH8.0, 1mM EDTA) and used in a PCR with primers MM407 and MM408. The primers are separated by 518 nucleotides in vaccinia WR and yield a fragment containing the N terminus of the thymidine kinase gene. The products were electrophoresed through a 2% agarose gel. The leftmost sample contains phiX 174 HaeIII digestion products; all



5 others contain the PCR product using primers MM407 and  
MM408 with the DNA sample indicated above the well

10 Figure 13. Promoter strength of recombinant viruses. The  
units of  $\beta$ -glu activity were determined as described by  
5 Miller (10) as adapted for 96-well plates. The  $A_{405}$  values  
were determined on a microplate reader (Dynatech MR3000)  
15 and the  $\beta$ -glu activity was determined by comparison to  $\beta$ -  
glu (Clontech) standards analyzed in the same assay.

20 Figure 14. Plaque assay on vEL/tk. Ten-fold dilutions of  
vEL/tk were incubated with Hutk<sup>-</sup> cells (top to bottom) for  
one hour at 37°C in 1ml of E-MEM (Gibco) with 10% Fetal  
Bovine Serum for one hour, the media was replaced with 3ml  
25 of E-MEM with 5% methyl cellulose (Sigma M-0387), 5% Fetal  
Bovine Serum and HAT supplement (Gibco), 25 or 125mM  
15 bromodeoxyuridine, or no drug, incubated for 48 hours at  
37°C, and stained with 0.5% Crystal Violet (Sigma C 0775),  
20% ethanol, 7.5% formaldehyde.

## 30 5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods for the  
20 identification of target antigens recognized by CTLs, and  
the use of such antigens in immunogenic compositions or  
35 vaccines to induce a cell-mediated immune response against  
cells which express the target antigens.

In one embodiment of the invention, tumor-  
40 specific CTLs generated in animals are used to screen  
25 expression libraries generated from tumor cell DNA, RNA or  
cDNA to identify reactive target antigens. To this end,  
animals tolerized with a non-tumorigenic human cell line  
are immunized with tumor cells derived from the non-  
45 tumorigenic cell line. The resulting CTLs, which are  
tumor-specific and not cross-reactive with normal cells,  
30 can be used to screen expression libraries constructed from  
tumor-cell derived DNA, RNA or cDNA. Clones so identified

5 in the library encode target antigens which are candidates  
for the immunogenic compositions and vaccines of the  
invention. Improved and modified vaccinia virus vectors  
10 for efficient construction of such DNA libraries using a  
"trimolecular recombination" approach are described to  
5 improve screening efficiency.

It is a preferred embodiment of the invention to  
tolerize animals, such as normal or transgenic mice, with  
15 normal human cells prior to immunizing with human tumor  
cells. Tolerance induction is preferred because the  
animal's immune response would otherwise be dominated by  
10 specificity for a large number of broadly expressed human  
proteins that are not specifically associated with tumor  
transformation. In a particularly preferred embodiment,  
and to enhance the efficiency of this approach, it is  
convenient to work with human tumors that are derived from  
25 an immortalized, non-tumorigenic human cell line by in  
15 vitro carcinogenesis or oncogene transformation. This  
provides a ready source of the normal control cells for an  
extended tolerization protocol in both neonatal and adult  
30 mice. For example, CTLs generated by this approach (see  
Section 7 below) can be employed in a selection procedure  
(such as that described in Section 8 below) to isolate  
20 recombinant clones that encode the target antigens from a  
tumor cDNA library, for example, such as that constructed  
35 in vaccinia virus by tri-molecular recombination (see  
Section 6 below).

In another embodiment of the invention, the  
40 products of genes that are differentially expressed in  
25 tumor cells are used to generate HLA-restricted CTLs which  
are evaluated for activity against authentic tumor cells.  
It is particularly preferred if methods such as  
45 Representational Difference Analysis (RDA) and differential  
display are employed to identify gene fragments that are  
differentially expressed in tumor versus normal cells.  
30 Conveniently, if it is determined that these gene products

5 are broadly expressed in other related tumors (see, for  
example, Sections 10 and 11 below), they may be used to  
select longer clones from the library (see, for example,  
10 Section 9.5) which may be tested for the ability to induce  
a tumor-specific immune response in, for example, human CD8  
5 and HLA transgenic mice (see, for example, Section 12).  
Gene products which generate tumor-specific cell mediated  
immunity are also candidates for the immunogenic  
15 compositions and vaccines of the invention. Improved  
methods for differential display are described that enhance  
screening efficiency by reducing false positives, and  
20 enhance the efficiency for isolating full length cDNAs.

The antigens identified using any of the  
foregoing strategies can be produced in quantity by  
recombinant DNA methods and formulated with an adjuvant  
that promotes a cell-mediated immune response. Preferably,  
25 the DNA encoding the target antigen is engineered into a  
recombinant virus that can be used to vaccinate animal  
15 hosts, including humans. In this regard, improved direct  
ligation vaccinia vectors are described that can be used to  
generate vaccines.

Another therapeutic strategy of the invention is  
to design vaccines that target a small set of HLA class I  
20 molecules which are expressed at elevated frequencies  
across ethnic populations. Extensive characterization of  
35 peptide binding motifs of different human class I MHC  
molecules has suggested that there are four major subtypes  
of HLA-A and HLA-B alleles (Sidney, J., et al., 1996,  
40 Immunol. Today 17:261-266) such that many peptides will  
bind to multiple members of a single group. The present  
25 invention also provides methods to target vaccines for  
patients based on their membership in a class I MHC group.  
In specific embodiments, class I MHC subtypes A2, A3, B7  
45 and B44 are targeted. Each group has an average  
representation across ethnic populations of between 40% and  
30 50%. It is estimated that the combination of all four  
groups (which include 50% to 60% of all known HLA-A and  
50

5 HLA-B alleles) covers 95% of the human population. In a  
specific embodiment, HLA-A2.1, the most frequently  
expressed HLA allele in human populations (Caucasian 43%,  
10 Black 20%, Chinese 25%) and the dominant member of the A2  
subtype, is targeted.

5 Although the methods of the invention described  
are used to identify reactive target antigens in tumor  
cells, the methods may also be used to identify target  
15 antigens in other target cells against which it is  
desirable to induce cell-mediated immunity. For example,  
the differential immunogenicity methods of the invention  
20 can be applied to identify immunogenic molecules of cells  
infected with virus, fungus or mycobacteria by tolerization  
of mice with uninfected cells followed by immunization with  
infected cells at different times after infection. The  
isolated CTLs can be employed to select recombinants that  
25 encode target antigens in a plasmid or viral expression  
library. An expression library can be constructed with  
cDNA isolated from the infected cell in a vaccinia virus  
vector using tri-molecular recombination.

30 A particular advantage of this approach is that  
it will identify potential antigens expressed not only by  
the pathogen but also by the host cell whose gene  
20 expression is altered as a result of infection. Since many  
35 pathogens elude immune surveillance by frequent  
reproduction and mutation, it may be of considerable value  
to develop a vaccine that targets host gene products that  
are not likely to be subject to mutation.

40 The differential gene expression strategies of the  
25 present invention may also be applied to identify  
immunogenic molecules of cells infected with virus, fungus  
or mycobacteria. More stable and/or previously  
45 unidentified antigens encoded by genes of either pathogen  
or host, including those which might remain cryptic without  
prior specific vaccination, may be identified.

30 Pathogens include, but are not limited to:

5 viral pathogens, such as human immunodeficiency virus,  
Epstein Barr virus, hepatitis virus, herpes virus, human  
papillomavirus, cytomegalovirus, respiratory syncytial  
10 virus; fungal pathogens, such as Candida albicans,  
pneumocystis carinii; and mycobacterial pathogens, such as  
5 M. tuberculosis, M. avium.

The following details and examples mention  
primarily target antigens in tumor cells. As will be  
15 appreciated from the foregoing, the methods of the  
invention may be adapted to identify target antigens in  
other target cells, such as virally infected cells, and may  
10 be useful in developing vaccines.

#### 5.1. IDENTIFYING TARGET ANTIGENS FOR USE IN VACCINES

The subsections below describe two strategies  
25 that can be used to identify target antigens or epitopes  
15 that are candidates for use in immunogenic formulations or  
vaccines. The two strategies described herein may be  
applied to identify target epitopes which include, but are  
30 not limited to, tumor specific, epitopes specific to a cell  
infected with a virus, fungus or mycobacteria, and/or  
epitopes specific to an autoimmune disease.  
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##### 5.1.1. INDUCTION OF CYTOTOXIC T LYMPHOCYTES SPECIFIC FOR HUMAN TUMORS AND THEIR USE TO SELECT DNA RECOMBINANTS THAT 35 ENCODE TARGET EPITOPES

40 In this embodiment of the invention, cytotoxic T  
25 cells specific for human tumors are induced in animals  
which have been tolerized with a non-tumorigenic,  
immortalized normal human cell line that does not express  
45 costimulator activity. These animals are subsequently  
immunized with costimulator transfected (e.g., B7  
transfected) tumor cells derived by in vitro mutagenesis or  
30 oncogene transformation from that same normal immortalized

5 human cell line. An alternative source of matched normal  
and tumor cell pairs that could be employed in this same  
fashion is to derive normal and tumor cell lines from  
10 different tissue samples of the same patient. For purposes  
of immunization, costimulator activity could also be  
5 introduced in these tumor cells by transfection with murine  
B7. This immunization regimen gives rise to tumor-specific  
CTL that are not crossreactive on the homologous normal  
15 cells. The primary purpose of inducing tumor-specific CTL  
is that they can be employed, as described below, to select  
for clones of recombinant tumor DNA that encode the target  
10 antigen. Such antigens, because they are differentially  
immunogenic in tumor as compared to normal cells, are  
20 candidates for immunogenic formulations or vaccines.  
Mammals of different species, most commonly diverse strains  
of inbred mice, can be employed for this purpose. Whether  
25 a particular formulation or vaccine is immunogenic in any  
15 particular individual will depend on whether specific  
peptides derived from that antigen can be processed and  
presented in association with the particular MHC molecules  
30 expressed by that individual. To narrow the focus of this  
selection process to antigens from which peptides can be  
derived that associate with a particular human HLA  
20 molecule, it is possible, as described in Section 7, to  
derive directly HLA restricted CTL from HLA and human CD8  
35 transgenic mice. Alternatively, differentially immunogenic  
molecules of the human tumor can be initially identified  
employing tumor-specific CTL restricted to any animal MHC.  
40 Antigens so identified can subsequently be characterized  
25 for the ability to be processed and presented in  
association with different human HLA types by primary in  
vitro stimulation of human peripheral blood lymphocytes  
45 (PBL), or, as described in Section 12, by immunization of  
HLA and human CD8 transgenic mice. The HLA transgene  
30 permits selection of a high affinity, HLA- restricted T  
cell repertoire in the mouse thymus. In addition, a human

5 CD8 transgene is most preferable because murine CD8 does not interact efficiently with human class I MHC.

10 The method to determine differential immunogenicity can be carried out in normal mice if genes encoding mouse MHC molecules are introduced into the human  
5 cell lines by transfection (Kriegler, M., 1991, Gene transfer and expression: A laboratory manual, W.H. Freeman and Co., N.Y.). Alternatively, antigens of the human cell  
15 lines may be re-presented by murine professional antigen presenting cells *in vivo* (Huang, et al., 1994, Science, 264:961-965) and *in vitro* (Inaba, et al., 1992, J. Exp.  
10 Med. 176:1702; Inaba, et al., 1993, J. Exp. Med. 178:479-488). To induce T cell tolerance during re-presentation of human antigens by murine dendritic cells it may be  
20 necessary to block costimulator activity with anti-B7.1 and anti-B7.2 antibodies. Specificity of the CTL generated in  
25 this way may be determined by comparing lysis of human tumor and normal target cells that have been transfected with HLA class I or that have been infected with HLA class I or that have been infected with HLA class I recombinant  
30 vaccinia virus.

35 Since immunogenicity of antigen in any individual depends on whether peptides derived from the antigen can be presented to T cells in association with MHC molecules of that particular individual, it may be separately determined by immunization of human volunteers or of human CD8 and HLA transgenic mice, which human HLA molecules are able to  
40 present peptides of any identified antigen. The two issues of immunogenicity and HLA associated presentation can be  
25 addressed simultaneously if HLA transgenic mice rather than normal mice are employed in the initial immunization.

45 The construction of transgenic mice is well known in the art and is described, for example, in Manipulating the Mouse Embryo: A laboratory Manual, Hogan, et al., Cold Spring Harbor Press, second edition, 1994. Human CD8  
30 transgenic mice may be constructed by the method of LaFace,

5 et al., J. Exp. Med. 182: 1315-25 (1995). Construction of  
new lines of transgenic mice expressing the human CD8alpha  
and CD8beta subunits may be made by insertion of the  
10 corresponding human cDNA into a human CD2 minigene based  
vector for T cell-specific expression in transgenic mice  
5 (Zhumabekov, et al., J. Immunol. Methods 185:133-140  
(1995)). HLA class I transgenic mice may be constructed by  
the methods of Chamberlain, et al., Proc. Natl. Acad. Sci.  
15 USA 85:7690-7694 (1988) or Bernhard, et al., J. Exp. Med.  
168: 1157-62 (1988) or Vitiello, et al., J. Exp. Med. 173:  
1007-1015 (1991) or Barra, et al., J. Immunol. 150: 3681-9  
10 (1993).

20 Construction of additional HLA class I transgenic  
mice may be achieved by construction of an H-2Kb cassette  
that includes 2 kb of upstream regulatory region together  
with the first two introns previously implicated in gene  
25 regulation (Kralova, et al., 1992, EMBO J. 11: 4591-4600).  
15 Endogenous translational start sites are eliminated from  
this region and restriction sites for insertion of HLA cDNA  
are introduced into the third exon followed by a polyA  
30 addition site. By including an additional 3kb of genomic  
H-2Kb sequence at the 3' end of this construct, the class I  
gene can be targeted for homologous recombination at the H-  
20 2Kb locus in embryonic stem cells. This has the advantage  
35 that the transgene is likely to be expressed at a defined  
locus known to be compatible with murine class I expression  
and that these mice are likely to be deficient for possible  
competition by H-2Kb expression at the cell membrane. It  
40 is believed that this will give relatively reproducible  
25 expression of diverse human HLA class I cDNA introduced in  
the same construct.

45 Most preferably, the tumor cell lines are a panel  
of tumor cell lines that are all derived from a single  
immortalized, non-tumorigenic cell line. Non-tumorigenic  
cells are most preferable for inducing tolerance to the  
30 large number of normal human proteins that are also  
50 expressed in tumor cells.



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Preferably, screening is performed on such a panel of tumor cell lines, independently derived from the same normal cells by diverse carcinogens or oncogene transformation. Screening of such a panel of tumor cell lines makes it possible to filter out antigenic changes that are carcinogen specific or that may arise by random genetic drift during in vitro propagation of a tumor cell line.

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The tumor-specific CTLs generated as described above can be used to screen expression libraries prepared from the target tumor cells in order to identify clones encoding the target epitope. DNA libraries constructed in a viral vector infectious for mammalian cells as described herein can be employed for the efficient selection of specific recombinants by CTLs. Major advantages of these infectious viral vectors are 1) the ease and efficiency with which recombinants can be introduced and expressed in mammalian cells, and 2) efficient processing and presentation of recombinant gene products in association with MHC molecules of the infected cell. At a low multiplicity of infection (m.o.i.), many target cells will express a single recombinant which is amplified within a few hours during the natural course of infection.

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In one embodiment of the invention, a representative DNA library is constructed in vaccinia virus. Preferably, a tri-molecular recombination method employing modified vaccinia virus vectors and related transfer plasmids is used to construct the representative DNA library in vaccinia virus. This method generates close to 100% recombinant vaccinia virus (see Section 6, Section 6.2 and 6.3).

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In a preferred embodiment (see also Section 14, Section 14.1.1), a vaccinia virus transfer plasmid pJ/K, a pUC 13 derived plasmid with a vaccinia virus thymidine kinase gene containing an in-frame Not I site, is further modified to incorporate one of two strong vaccinia virus

5 promoters, e.g., either a 7.5K vaccinia virus promoter or a  
strong synthetic early/late (E/L) promoter, followed by Not  
I and Apa I restriction sites. The Apa I site is  
10 preferably preceded by a strong translational initiation  
sequence including the ATG codon. This modification is  
5 preferably introduced within the vaccinia virus thymidine  
kinase (tk) gene so that it is flanked by regulatory and  
coding sequences of the viral tk gene. Each of the two  
15 modifications within the tk gene of a plasmid vector may be  
transferred by homologous recombination in the flanking tk  
10 sequences into the genome of the Vaccinia Virus WR strain  
derived vNotI<sup>-</sup> vector to generate two new viral vectors.  
20 Importantly, following Not I and Apa I restriction  
endonuclease digestion of these two viral vectors, two  
large viral DNA fragments can be isolated each including a  
separate non-homologous segment of the vaccinia tk gene and  
25 together comprising all the genes required for assembly of  
infectious viral particles.

30 In one embodiment, such modifications are  
introduced in the Modified Virus Ankara (MVA) strain of  
vaccinia, which is replication deficient in mammalian cells  
(Meyer, et al., 1991. J. Gen. Virol. 72:1031-1038).

20 In a preferred embodiment, the following method  
35 is used to enrich for, and select for those cells infected  
with the recombinant viruses that express the target  
epitopes of specific cytotoxic T cells. An adherent  
monolayer of cells is infected with a recombinant viral  
40 library, e.g. a vaccinia recombinant viral library, at  
25 m.o.i. less than or equal to 1. It is important that these  
cells do not themselves express the target epitopes  
recognized by specific CTLs but that these epitopes are  
45 represented in the viral library. In addition, for  
selection by CTLs, the infected cells must express an  
appropriate MHC molecule that can associate with and  
30 present the target peptide to T cells.

5 After 12 hours infection with recombinant virus,  
the monolayer is washed to remove any non-adherent cells.  
CTLs of defined specificity are added for 30 min. During  
10 this time, some of the adherent cells infected with a  
recombinant particle that leads to expression of the target  
5 epitope will interact with a specific CTL and undergo a  
lytic event. Cells that undergo a lytic event are released  
15 from the monolayer and can be harvested in the floating  
cell population. The above-described protocol is repeated  
for preferably five or more cycles, to increase the level  
of enrichment obtained by this procedure.

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20 5.1.2. SCREENING CYTOTOXIC LYMPHOCYTES  
GENERATED AGAINST PRODUCTS OF  
GENES DIFFERENTIALLY EXPRESSED  
IN TUMOR CELLS FOR ACTIVITY  
25 AGAINST AUTHENTIC TUMOR CELLS

15 In this embodiment of the invention, the products  
of genes that are differentially expressed in a tumor are  
used to generate HLA-restricted CTLs (e.g., by immunization  
30 of transgenic animals or in vitro stimulation of human PBL  
with antigen presenting cells that express the appropriate  
MHC ). The CTLs so generated are assayed for activity  
20 against authentic tumor cells in order to identify the  
35 differentially expressed gene which encodes the effective  
target epitope.

In essence, this approach to identify tumor-  
specific antigens is the reverse of the strategy described  
40 in the preceding section. Rather than isolating CTLs  
25 generated against authentic tumor cells to screen  
expression libraries of tumor-specific cDNA, the tumor-  
specific cDNA or gene products (i.e., the product of genes  
45 differentially expressed in tumors) are used to generate  
CTLs which are then screened using authentic tumor. This  
strategy is quite advantageously used to identify target  
30 epitopes for many human tumor types where it has not been  
50 possible to generate tumor-specific CTL directly from

5 patients. This strategy provides an additional advantage  
in that cryptic tumor antigens can be identified. Rather  
than only assaying for what is immunogenic on a tumor cell,  
10 this embodiment of the invention allows for the evaluation  
and assessment of tumor cell products that can become  
5 immunogenic if the representation of tumor-specific T cells  
is first augmented by vaccination.

15 Differentially expressed genes derived from the  
tumor can be identified using standard techniques well  
known to those skilled in the art (e.g., see Liang &  
Pardee, 1992, Science 257:967-971, which is incorporated by  
20 reference herein in its entirety). Preferably, the  
improved differential display methods described in Sections  
9.2 and 9.3, infra, may be used to reduce false positives  
and enhance the efficiency for isolating full length cDNAs  
25 corresponding to the identified DNA fragments. Each  
differentially expressed gene product is potentially  
15 immunogenic, and may be represented as a low-abundance or  
high abundance transcript.

30 In order to identify the differentially expressed  
gene products that might be candidates for tumor  
immunotherapy, it is necessary to have a means of  
delivering the product for immunization in an environment  
20 in which T cell responses to peptides associated with human  
35 HLA can be induced. To this end, the differentially  
expressed cDNA is incorporated into an expression vector,  
preferably a viral vector (such as the vaccinia vectors  
described herein) so that quantities of the gene product  
40 adequate for immunization are produced. Immunization can  
25 be accomplished using the recombinantly expressed gene  
product formulated in a subunit vaccine (e.g., mixed with a  
suitable adjuvant that can promote a cell mediated immune  
45 response). Preferably a recombinant viral expression  
vector, such as vaccinia, can be used to immunize (Bennock  
& Yewdell, 1990, Current Topics In Microbiol. and Immunol.  
30 163:153-178). Most preferably, transgenic mice are  
employed which express a human class I MHC molecule, so  
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5 that HLA-restricted murine cytotoxic T cells specific for  
the gene product can be induced and isolated (Shirai, M.,  
et al., 1995, J. Immunol. 154:2733-42; Wentworth et al.,  
10 1996, Eur. J. of Immunol. 26:97-101). Alternatively, human  
PBL are stimulated in vitro with antigen presenting cells  
5 that express homologous HLA.

The significance of HLA compatibility is that T  
15 cells recognize peptides that bind to, and are transported  
to the surface of antigen presenting cells in association  
with major histocompatibility molecules. T cells of HLA  
transgenic mice are, therefore, primed to recognize a  
10 specific peptide in association with the expressed human  
HLA and crossreactivity with human tumor cells depends on  
expression of that same tumor peptide in association with  
the same HLA molecule.

25 The CTLs induced by the immunization can be  
tested for cross reactivity on HLA compatible tumors that  
15 express the corresponding mRNA. The CTLs can be assayed  
for their ability to kill authentic tumor cells *in vitro* or  
*in vivo*. To this end, assays described in Section 7 can be  
30 used, or other similar assays for determining tumor cell  
specificity and killing which are well known to those  
20 skilled in the art.

35 Using this approach, target epitopes which are  
particularly good candidates for tumor immunotherapy in  
human patients are identified as those which meet the  
following criteria: (a) the gene is differentially  
40 expressed in multiple human tumors; (b) the gene products  
25 are immunogenic in association with HLA; and (c) the  
specific CTLs induced are cross reactive on human tumor  
cells.

## 5.2. VACCINE FORMULATIONS

The present invention encompasses the expression of the identified target epitope in either eucaryotic or procaryotic recombinant expression vectors; and the formulation of the identified epitope as immunogenic and/or antigenic compositions. In accordance with the present invention, the recombinantly expressed target epitope may be expressed, purified and formulated as a subunit vaccine. The identified target epitope may also be constructed into viral vectors for use in vaccines. In this regard, either a live recombinant viral vaccine, an inactivated recombinant viral vaccine, or a killed recombinant viral vaccine can be formulated.

### 5.2.1. EXPRESSION OF THE TARGET EPITOPE IN PROCARYOTIC AND EUCARYOTIC EXPRESSION SYSTEMS

The present invention encompasses expression systems, both eucaryotic and procaryotic expression vectors, which may be used to express the identified target epitope. The identified epitope may be expressed in both truncated or full-length forms of the epitope, in particular for the formation of subunit vaccines.

The present invention encompasses the expression of nucleotide sequences encoding the identified epitopes and immunologically equivalent fragments. Such immunologically equivalent fragments may be identified by making analogs of the nucleotide sequence encoding the identified epitopes that are truncated at the 5' and/or 3' ends of the sequence and/or have one or more internal deletions, expressing the analog nucleotide sequences, and determining whether the resulting fragments immunologically are recognized by the epitope specific CTLs and induce a cell-mediated immune response.

The invention encompasses the DNA expression vectors that contain any of the foregoing coding sequences

operatively associated with a regulatory element that directs expression of the coding sequences and genetically engineered host cells that contain any of the foregoing coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell. As used herein, regulatory elements include but are not limited to, inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression.

The target epitope gene products or peptide fragments thereof, may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing the epitope gene polypeptides and peptides of the invention by expressing nucleic acid containing epitope gene sequences are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing epitope gene product coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. See, for example, the techniques described in Sambrook et al., 1989, supra, and Ausubel et al., 1989, supra. Alternatively, RNA capable of encoding glycoprotein epitope gene product sequences may be chemically synthesized using, for example, synthesizers. See, for example, the techniques described in "Oligonucleotide Synthesis", 1984, Gait, M.J. ed., IRL Press, Oxford, which is incorporated by reference herein in its entirety.

The invention also encompasses nucleotide sequences that encode peptide fragments of the identified epitope gene products. For example, polypeptides or peptides corresponding to the extracellular domain of the selected epitope may be useful as "soluble" protein which would facilitate secretion, particularly useful in the

5 production of subunit vaccines. The selected epitope gene  
product or peptide fragments thereof, can be linked to a  
heterologous epitope that is recognized by a commercially  
10 available antibody is also included in the invention. A  
durable fusion protein may also be engineered; i.e., a  
5 fusion protein which has a cleavage site located between  
the selected epitope sequence and the heterologous protein  
sequence, so that the selected epitope can be cleaved away  
15 from the heterologous moiety. For example, a collagenase  
cleavage recognition consensus sequence may be engineered  
between the selected epitope protein or peptide and the  
10 heterologous peptide or protein. The epitopic domain can  
be released from this fusion protein by treatment with  
collagenase. In a preferred embodiment of the invention, a  
fusion protein of glutathione-S-transferase and the  
selected epitope protein may be engineered.

25 The selected epitope proteins of the present  
15 invention for use in vaccine preparations, in particular  
subunit vaccine preparations, are substantially pure or  
homogeneous. The protein is considered substantially pure  
or homogeneous when at least 60 to 75% of the sample  
30 exhibits a single polypeptide sequence. A substantially  
pure protein will preferably comprise 60 to 90% of a  
20 protein sample, more preferably about 95% and most  
preferably 99%. Methods which are well known to those  
35 skilled in the art can be used to determine protein purity  
or homogeneity, such as polyacrylamide gel electrophoresis  
of a sample, followed by visualizing a single polypeptide  
40 band on a staining gel. Higher resolution may be  
25 determined using HPLC or other similar methods well known  
in the art.

45 The present invention encompasses polypeptides  
which are typically purified from host cells expressing  
recombinant nucleotide sequences encoding these proteins.  
Such protein purification can be accomplished by a variety  
30 of methods well known in the art. In a preferred  
50 embodiment, the epitope protein of the present invention is



5 expressed as a fusion protein with glutathione-S-  
transferase. The resulting recombinant fusion proteins  
10 purified by affinity chromatography and the epitope protein  
domain is cleaved away from the heterologous moiety  
5 resulting in a substantially pure protein sample. Other  
methods known to those skilled in the art may be used; see  
for example, the techniques described in "Methods In  
15 Enzymology", 1990, Academic Press, Inc., San Diego,  
"Protein Purification: Principles and Practice", 1982,  
Springer-Verlag, New York, which are incorporated by  
reference herein in their entirety.

20 5.2.1.1. EUCARYOTIC AND PROCARYOTIC EXPRESSION  
VECTORS

25 The present invention encompasses expression  
systems, both eucaryotic and procaryotic expression  
vectors, which may be used to express the selected epitope.  
15 A variety of host-expression vector systems may be utilized  
to express the selected target epitope gene of the  
invention. Such host-expression systems represent vehicles  
30 by which the coding sequences of interest may be produced  
and subsequently purified, but also represent cells which  
may, when transformed or transfected with the appropriate  
20 nucleotide coding sequences, exhibit the selected epitope  
gene product of the invention in situ. These include but  
35 are not limited to microorganisms such as bacteria (e.g.,  
E. coli, B. subtilis) transformed with recombinant  
bacteriophage DNA, plasmid DNA or cosmid DNA expression  
40 vectors containing selected epitope gene product coding  
25 sequences; yeast (e.g., Saccharomyces, Pichia) transformed  
with recombinant yeast expression vectors containing the  
selected epitope gene product coding sequences; insect cell  
45 systems infected with recombinant virus expression vectors  
(e.g., baculovirus) containing the selected epitope gene  
30 product coding sequences; plant cell systems infected with  
recombinant virus expression vectors (e.g., cauliflower

5 mosaic virus, CaMV; tobacco mosaic virus, TMV) or  
transformed with recombinant plasmid expression vectors  
(e.g., Ti plasmid) containing selected epitope gene product  
10 coding sequences; or mammalian cell systems (e.g., COS,  
CHO, BHK, 293, 3T3) harboring recombinant expression  
5 constructs containing promoters derived from the genome of  
mammalian cells (e.g., metallothionein promoter) or from  
mammalian viruses (e.g., the adenovirus late promoter; the  
15 vaccinia virus 7.5K promoter).

#### 10 5.2.1.2. HOST CELLS

20 The present invention encompasses the expression  
of the selected epitope in animal and insect cell lines.  
In a preferred embodiment of the present invention, the  
selected epitope is expressed in a baculovirus vector in an  
insect cell line to produce an unglycosylated antigen. In  
25 another preferred embodiment of the invention, the selected  
15 epitope is expressed in a stably transfected mammalian host  
cell, e.g., T lymphocyte cell line to produce a  
glycosylated antigen. The selected epitopes which are  
expressed recombinantly by these cell lines may be  
30 formulated as subunit vaccines.

20 A host cell strain may be chosen which modulates  
the expression of the inserted sequences, or modifies and  
35 processes the gene product in the specific fashion desired.  
Such modifications (e.g., glycosylation) and processing  
(e.g. cleavage) of protein products may be important for  
the function of the protein. Different host cells have  
40 characteristic and specific mechanisms for the post-  
25 translational processing and modification of proteins and  
gene products. Appropriate cell lines or host systems can  
be chosen to ensure the correct modification of the foreign  
45 protein expressed. To this end, eucaryotic host cells  
which possess the cellular machinery for proper processing  
of the primary transcript, glycosylation, and  
30 phosphorylation of the gene product may be used. Such

5 mammalian host cells include but are not limited to CHO,  
VERO, BHK, HeLa, COS, MDCK, 293, 3T3 and WI38 cell lines.

10 For long term, high-yield production of  
recombinant proteins, stable expression is preferred. For  
example, cell lines which stably express the selected  
5 target epitope may be engineered. Rather than using  
expression vectors which contain viral origins of  
replication, host cells can be transformed with DNA  
15 controlled by appropriate expression control elements  
(e.g., promoter, enhancer, sequences, transcription  
terminators, polyadenylation sites, etc.), and a selectable  
20 marker. Following the introduction of the foreign DNA,  
engineered cells may be allowed to grow for 1-2 days in an  
enriched media, and then are switched to a selective media.  
The selectable marker in the recombinant plasmid confers  
resistance to the selection and allows cells to stably  
25 integrate the plasmid into their chromosomes and grow to  
form foci which in turn can be cloned and expanded into  
cell lines. This method may advantageously be used to  
engineer cell lines. This method may advantageously be  
30 used to engineer cell lines which express the selected  
epitope gene products. Such cell lines would be  
particularly useful in screening and evaluation of  
20 compounds that affect the endogenous activity of the  
selected epitope gene product.

35 A number of selection systems may be used,  
including but not limited to the herpes simplex virus  
thymidine kinase (Wigler, et al., 1977, Cell 11:223),  
40 hypoxanthine-guanine phosphoribosyltransferase (Szybalska &  
Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and  
25 adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell  
22:817) genes can be employed in tk<sup>-</sup>, hgp<sup>+</sup> or aprt<sup>-</sup> cells,  
45 respectively. Also, antimetabolite resistance can be used  
as the basis of selection for the following genes: dhfr,  
which confers resistance to methotrexate (Wigler, et al.,  
30 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981,  
Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers

5 resistance to mycophenolic acid (Mulligan & Berg, 1981,  
Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers  
10 resistance to the aminoglycoside G-418 (Colberre-Garapin,  
et al., 1981, J. Mol. Biol. 150:1); and hygro, which  
confers resistance to hygromycin (Santerre, et al., 1984,  
5 Gene 30:147).

Alternatively, any fusion protein may be readily  
15 purified by utilizing an antibody specific for the fusion  
protein being expressed. For example, a system described  
by Janknecht et al. allows for the ready purification of  
non-denatured fusion proteins expressed in human cell lines  
20 (Janknecht, et al., 1991, Proc. Natl. Acad. Sci. USA 88:  
8972-8976). In this system, the gene of interest is  
subcloned into a vaccinia recombination plasmid such that  
the gene's open reading frame is translationally fused to  
an amino-terminal tag consisting of six histidine residues.  
25 Extracts from cells infected with recombinant vaccinia  
virus are loaded onto Ni<sup>2+</sup>-nitriloacetic acid-agarose  
columns and histidine-tagged proteins are selectively  
eluted with imidazole-containing buffers.

#### 5.2.2. EXPRESSION OF TARGET EPITOPE IN RECOMBINANT VIRAL VACCINES

20 In another embodiment of the present invention,  
35 either a live recombinant viral vaccine or an inactivated  
recombinant viral vaccine expressing the selected target  
epitope can be engineered. A live vaccine may be preferred  
because multiplication in the host leads to a prolonged  
40 stimulus of similar kind and magnitude to that occurring in  
25 natural infections, and therefore, confers substantial,  
long-lasting immunity. Production of such live  
recombinant virus vaccine formulations may be accomplished  
45 using conventional methods involving propagation of the  
virus in cell culture or in the allantois of the chick  
embryo followed by purification.  
30

5 In this regard, a variety of viruses may be  
genetically engineered to express the selected epitope.  
For vaccine purposes, it may be required that the  
10 recombinant viruses display attenuation characteristics.  
Current live virus vaccine candidates for use in humans are  
5 either cold adapted, temperature sensitive, or attenuated.  
The introduction of appropriate mutations (e.g., deletions)  
15 into the templates used for transfection may provide the  
novel viruses with attenuation characteristics. For  
example, specific multiple missense mutations that are  
associated with temperature sensitivity or cold adaptation  
20 can be made into deletion mutations and/or multiple  
mutations can be introduced into individual viral genes.  
These mutants should be more stable than the cold or  
temperature sensitive mutants containing single point  
25 mutations and reversion frequencies should be extremely  
low. Alternatively, recombinant viruses with "suicide"  
15 characteristics may be constructed. Such viruses go  
through only one or a few rounds of replication in the  
host.

30 For purposes of the invention, any virus may be  
used in accordance with the present invention which: (a)  
displays an attenuated phenotype or may be engineered to  
20 display attenuated characteristics; (b) displays a tropism  
35 for mammals, in particular humans, or may be engineered to  
display such a tropism; and (c) may be engineered to  
express the selected target epitope of the present  
invention.

40 Vaccinia viral vectors may be used in accordance  
25 with the present invention, as large fragments of DNA are  
easily cloned into its genome and recombinant attenuated  
vaccinia variants have been described (Meyer, et al., 1991,  
45 J. Gen. Virol. 72:1031-1038). Orthomyxoviruses, including  
influenza; Paramyxoviruses, including respiratory syncytial  
virus and Sendai virus; and Rhabdoviruses may be engineered  
30 to express mutations which result in attenuated phenotypes  
50 (see U.S. Patent Serial No. 5,578,473, issued November 26,

5 1996). These viral genomes may also be engineered to  
express foreign nucleotide sequences, such as the selected  
epitopes of the present invention (see U.S. Patent Serial  
10 No. 5,166,057, issued November 24, 1992, incorporated  
herein by reference in its entirety). Reverse genetic  
5 techniques can be applied to manipulate negative and  
positive strand RNA viral genomes to introduce mutations  
which result in attenuated phenotypes, as demonstrated in  
15 influenza virus, Herpes Simplex virus, cytomegalovirus and  
Epstein-Barr virus, Sindbis virus and poliovirus (see  
Palese et al., 1996, Proc. Natl. Acad. Sci. USA 93:11354-  
10 11358). These techniques may also be utilized to introduce  
foreign DNA, i.e., the selected target epitopes, to create  
recombinant viral vectors to be used as vaccines in  
accordance with the present invention. In addition,  
20 attenuated adenoviruses and retroviruses may be engineered  
to express the target epitope. Therefore, a wide variety  
25 of viruses may be engineered to design the vaccines of the  
present invention, however, by way of example, and not by  
limitation, recombinant attenuated vaccinia vectors  
30 expressing the selected target epitope for use as vaccines  
are described herein.

In one embodiment, a recombinant modified  
20 vaccinia variant, Modified Virus Ankara (MVA) is used in a  
vaccine formulation. This modified virus has been passaged  
35 for 500 cycles in avian cells and is unable to undergo a  
full infectious cycle in mammalian cells (Meyer, et al.,  
1991, J. Gen. Virol. 72:1031-1038). When used as a  
40 vaccine, the recombinant virus goes through a single  
25 replication cycle and induces a sufficient level of immune  
response but does not go further in the human host and  
cause disease. Recombinant viruses lacking one or more of  
45 essential vaccinia virus genes are not able to undergo  
successive rounds of replication. Such defective viruses  
can be produced by co-transfecting vaccinia vectors lacking  
30 a specific gene(s) required for viral replication into cell  
50 lines which permanently express this gene(s). Viruses

5 lacking an essential gene(s) will be replicated in these  
cell lines but when administered to the human host will not  
be able to complete a round of replication. Such  
10 preparations may transcribe and translate — in this  
abortive cycle — a sufficient number of genes to induce an  
5 immune response.

Alternatively, larger quantities of the strains  
can be administered, so that these preparations serve as  
15 inactivated (killed) virus, vaccines. For inactivated  
vaccines, it is preferred that the heterologous gene  
product be expressed as a viral component, so that the gene  
10 product is associated with the virion. The advantage of  
such preparations is that they contain native proteins and  
do not undergo inactivation by treatment with formalin or  
20 other agents used in the manufacturing of killed virus  
vaccines.

In another embodiment of the invention,  
15 inactivated vaccine formulations are prepared using  
conventional techniques to "kill" the recombinant viruses.  
Inactivated vaccines are "dead" in the sense that their  
30 infectivity has been destroyed. Ideally, the infectivity  
of the virus is destroyed without affecting immunogenicity.  
In order to prepare inactivated vaccines, the recombinant  
20 virus may be grown in cell culture or in the allantois of  
the chick embryo, purified by zonal ultracentrifugation,  
35 inactivated by formaldehyde or  $\beta$ -propiolactone, and pooled.  
The resulting vaccine is usually inoculated  
intramuscularly.

Inactivated viruses may be formulated with a  
25 suitable adjuvant in order to enhance the immunological  
response. Such adjuvants may include but are not limited  
to mineral gels, e.g., aluminum hydroxide; surface active  
45 substances such as lysolecithin, pluronic polyols,  
polyanions; peptides; oil emulsions; and potentially useful  
human adjuvants such as BCG and Corynebacterium parvum.

30

5.2.3. METHODS OF TREATMENT AND/OR VACCINATION

Since the identified target epitopes of the present invention can be produced in large amounts, the antigen thus produced and purified has use in vaccine preparations. The target epitope may be formulated into a subunit vaccine preparation, or may be engineered into viral vectors and formulated into vaccine preparations. Alternatively, the DNA encoding the target epitope may be administered directly as a vaccine formulation. The "naked" plasmid DNA once administered to a subject invades cells, is expressed on the surface of the invaded cell and elicits a cellular immune response, so that T lymphocytes will attack cells displaying the selected epitope. The selected epitope also has utility in diagnostics, e.g., to detect or measure in a sample of body fluid from a subject the presence of tumors and thus to diagnose cancer and tumors and/or to monitor the cellular immune response of the subject subsequent to vaccination.

The recombinant viruses of the invention can be used to treat tumor-bearing mammals, including humans, to generate an immune response against the tumor cells. The generation of an adequate and appropriate immune response leads to tumor regression *in vivo*. Such "vaccines" can be used either alone or in combination with other therapeutic regimens, including but not limited to chemotherapy, radiation therapy, surgery, bone marrow transplantation, etc. for the treatment of tumors. For example, surgical or radiation techniques could be used to debulk the tumor mass, after which, the vaccine formulations of the invention can be administered to ensure the regression and prevent the progression of remaining tumor masses or micrometastases in the body. Alternatively, administration of the "vaccine" can precede such surgical, radiation or chemotherapeutic treatment.

Alternatively, the recombinant viruses of the invention can be used to immunize or "vaccinate" tumor-free



5 subjects to prevent tumor formation. With the advent of  
genetic testing, it is now possible to predict a subject's  
predisposition for cancers. Such subjects, therefore, may  
10 be immunized using a recombinant vaccinia virus expressing  
an appropriate tumor-associated antigen.

5 The immunopotency of the epitope vaccine  
formulations antigen can be determined by monitoring the  
immune response in test animals following immunization or  
15 by use of any immunoassay known in the art. Generation of  
a cell-mediated immune response may be taken as an  
indication of an immune response. Test animals may include  
10 mice, hamsters, dogs, cats, monkeys, rabbits, chimpanzees,  
etc., and eventually human subjects.

Suitable preparations of such vaccines include  
injectables, either as liquid solutions or suspensions;  
25 solid forms suitable for solution in, suspension in, liquid  
prior to injection, may also be prepared. The preparation  
15 may also be emulsified, or the polypeptides encapsulated in  
liposomes. the active immunogenic ingredients are often  
mixed with excipients which are pharmaceutically acceptable  
and compatible with the active ingredient. Suitable  
30 excipients are, for example, water, saline, dextrose,  
glycerol, ethanol, or the like and combinations thereof.  
20 In addition, if desired, the vaccine preparation may also  
include minor amounts of auxiliary substances such as  
35 wetting or emulsifying agents, pH buffering agents, and/or  
adjuvants which enhance the effectiveness of the vaccine.

Examples of adjuvants which may be effective,  
40 include, but are not limited to: aluminum hydroxide, N-  
25 acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-  
acetyl-nor-muramyl-L-alanyl-D-isoglutamine, N-  
acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-  
45 dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine,  
GM-CSF, QS-21 (investigational drug, Progenics  
Pharmaceuticals, Inc.), DETOX (investigational drug, Ribi  
30 Pharmaceuticals), and BCG.

5                   The effectiveness of an adjuvant may be  
determined by measuring the induction of the cellular  
immune response directed against the target epitope.

10                   The vaccines of the invention may be multivalent  
or univalent. Multivalent vaccines are made from  
5 recombinant viruses that direct the expression of more than  
one antigen.

15                   The composition, if desired, can also contain  
minor amounts of wetting or emulsifying agents, or pH  
buffering agents. The composition can be a liquid  
solution, suspension, emulsion, tablet, pill, capsule,  
10 sustained release formulation, or powder. Oral formulation  
can include standard carriers such as pharmaceutical grades  
of mannitol, lactose, starch, magnesium stearate, sodium  
saccharine, cellulose, magnesium carbonate, etc.

20                   Generally, the ingredients are supplied either  
separately or mixed together in unit dosage form, for  
15 example, as a dry lyophilized powder or water free  
concentrate in a hermetically sealed container such as an  
ampoule or sachette indicating the quantity of active  
30 agent. Where the composition is administered by injection,  
an ampoule of sterile diluent can be provided so that the  
ingredients may be mixed prior to administration.

20                   In a specific embodiment, a lyophilized epitope  
35 of the invention is provided in a first container; a second  
container comprises diluent consisting of an aqueous  
solution of 50% glycerin, 0.25% phenol, and an antiseptic  
(e.g., 0.005% brilliant green).

40                   Use of purified antigens as vaccine preparations  
25 can be carried out by standard methods. For example, the  
purified protein(s) should be adjusted to an appropriate  
concentration, formulated with any suitable vaccine  
45 adjuvant and packaged for use. Suitable adjuvants may  
include, but are not limited to: mineral gels, e.g.,  
30 aluminum hydroxide; surface active substances such as  
lysolecithin, pluronic polyols; polyanions; peptides; oil

5 emulsions; alum, and MDP. The immunogen may also be  
incorporated into liposomes, or conjugated to  
10 polysaccharides and/or other polymers for use in a vaccine  
formulation. In instances where the recombinant antigen is  
a hapten, i.e., a molecule that is antigenic in that it can  
5 react selectively with cognate antibodies, but not  
immunogenic in that it cannot elicit an immune response,  
15 the hapten may be covalently bound to a carrier or  
immunogenic molecule; for instance, a large protein such as  
serum albumin will confer immunogenicity to the hapten  
coupled to it. The hapten-carrier may be formulated for  
10 use as a vaccine.

20 Many methods may be used to introduce the vaccine  
formulations described above into a patient. These  
include, but are not limited to, oral, intradermal,  
25 intramuscular, intraperitoneal, intravenous, subcutaneous,  
intranasal, transdermal, epidural, pulmonary, gastric,  
15 intestinal, rectal, vaginal, or urethral routes. When the  
method of treatment uses a live recombinant vaccinia  
vaccine formulation of the invention, it may be preferable  
30 to introduce the formulation via the natural route of  
infection of the vaccinia virus, i.e., through a mucosal  
membrane or surface, such as an oral, nasal, gastric,  
20 intestinal, rectal, vaginal or urethral route. To induce a  
CTL response, the mucosal route of administration may be  
35 through an oral or nasal membrane. Alternatively, an  
intramuscular or intraperitoneal route of administration  
may be used. Preferably, a dose of  $10^6$  -  $10^7$  PFU (plaque  
40 forming units) of cold adapted recombinant vaccinia virus  
25 is given to a human patient.

The precise dose of vaccine preparation to be  
45 employed in the formulation will also depend on the route  
of administration, and the nature of the patient, and  
should be decided according to the judgment of the  
practitioner and each patient's circumstances according to  
30 standard clinical techniques. An effective immunizing

5 amount is that amount sufficient to produce an immune response to the antigen in the host to which the vaccine preparation is administered.

10 Where subsequent or booster doses are required, a modified vaccinia virus such as MVA can be selected as the  
5 parental virus used to generate the recombinant.

Alternatively, another virus, e.g., adenovirus, canary pox virus, or a subunit preparation can be used to boost.

15 Immunization and/or cancer immunotherapy may be accomplished using a combined immunization regimen, e.g., immunization with a recombinant vaccinia viral vaccine of  
10 the invention and a boost of a recombinant vaccinia viral vaccine. In such an embodiment, a strong secondary CD8<sup>+</sup> T cell response is induced after priming and boosting with different viruses expressing the same epitope (for such  
20 methods of immunization and boosting, see, e.g., Murata et al., Cellular Immunol. 173:96-107). For example, a patient  
25 is first primed with a vaccine formulation of the invention comprising a recombinant vaccinia virus expressing an epitope, e.g., a selected tumor-associated antigen or fragment thereof. The patient is then boosted, e.g., 21  
30 days later, with a vaccine formulation comprising a recombinant virus other than vaccinia expressing the same  
20 epitope. Such priming followed by boosting induces a strong secondary CD8<sup>+</sup> T cell response. Such a priming and  
35 boosting immunization regimen is preferably used to treat a patient with a tumor, metastasis or neoplastic growth expressing the selected tumor-associated antigen.

40 In yet another embodiment, the recombinant  
25 vaccinia viruses can be used as a booster immunization subsequent to a primary immunization with inactivated tumor cells, a subunit vaccine containing the tumor-associated  
45 antigen or its epitope, or another recombinant viral vaccine, e.g., adenovirus, canary pox virus, or MVA.

In an alternate embodiment, recombinant vaccinia  
30 virus encoding a particular tumor-associated antigen, epitope or fragment thereof may be used in adoptive  
50

5 immunotherapeutic methods for the activation of T  
lymphocytes that are histocompatible with the patient and  
specific for the tumor-associated antigen (for methods of  
10 adoptive immunotherapy, see, e.g., Rosenberg, U.S. Patent  
No. 4,690,915, issued September 1, 1987; Zarling, et al.,  
5 U.S. Patent No. 5,081,029, issued January 14, 1992). Such  
T lymphocytes may be isolated from the patient or a  
histocompatible donor. The T lymphocytes are activated in  
15 vitro by exposure to the recombinant vaccinia virus of the  
invention. Activated T lymphocytes are expanded and  
inoculated into the patient in order to transfer T cell  
20 immunity directed against the tumor-associated antigen  
epitope.

The invention also provides a pharmaceutical pack  
or kit comprising one or more containers comprising one or  
25 more of the ingredients of the vaccine formulations of the  
invention. Associated with such container(s) can be a  
15 notice in the form prescribed by a governmental agency  
regulating the manufacture, use or sale of pharmaceuticals  
or biological products, which notice reflects approval by  
30 the agency of manufacture, use or sale for human  
administration.

20 The invention will be better understood by  
reference to the specific embodiments detailed in the  
35 examples which follow.

6. **EXAMPLE: TRIMOLECULAR RECOMBINATION  
EMPLOYING MODIFIED VACCINIA VIRUS  
40 VECTORS TO MAKE EXPRESSION LIBRARIES**

25 This example describes a tri-molecular  
recombination method employing modified vaccinia virus  
vectors and related transfer plasmids that generates close  
45 to 100% recombinant vaccinia virus and, for the first time,  
allows efficient construction of a representative DNA  
30 library in vaccinia virus.

### 6.1. CONSTRUCTION OF THE VECTORS

The previously described vaccinia virus transfer plasmid pJ/K, a pUC 13 derived plasmid with a vaccinia virus thymidine kinase gene containing an in-frame Not I site (Merchlinisky, M. et al., Virology 190:522-526), was further modified to incorporate a strong vaccinia virus promoter followed by Not I and Apa I restriction sites. Two different vectors, p7.5/tk and pEL/tk, included, respectively, either the 7.5K vaccinia virus promoter or a strong synthetic early/late (E/L) promoter (Fig. 1). The Apa I site was preceded by a strong translational initiation sequence including the ATG codon. This modification was introduced within the vaccinia virus thymidine kinase (*tk*) gene so that it was flanked by regulatory and coding sequences of the viral *tk* gene. The modifications within the *tk* gene of these two new plasmid vectors were transferred by homologous recombination in the flanking *tk* sequences into the genome of the Vaccinia Virus WR strain derived vNotI<sup>-</sup> vector to generate new viral vectors v7.5/tk and vEL/tk. Importantly, following Not I and Apa I restriction endonuclease digestion of these viral vectors, two large viral DNA fragments were isolated each including a separate non-homologous segment of the vaccinia *tk* gene and together comprising all the genes required for assembly of infectious viral particles. Further details regarding the construction and characterization of these vectors and their alternative use for direct ligation of DNA fragments in vaccinia virus are described in section 14 infra.

### 6.2. GENERATION OF AN INCREASED FREQUENCY OF VACCINIA VIRUS RECOMBINANTS

Standard methods for generation of recombinants in vaccinia virus exploit homologous recombination between a recombinant vaccinia transfer plasmid and the viral

5 genome. Table 1 shows the results of a model experiment in  
which the frequency of homologous recombination following  
transfection of a recombinant transfer plasmid into  
10 vaccinia virus infected cells was assayed under standard  
conditions. To facilitate functional assays, a minigene  
5 encoding the immunodominant 257-264 peptide epitope of  
ovalbumin in association with H-2K<sup>b</sup> was inserted at the Not  
I site in the transfer plasmid tk gene. As a result of  
15 homologous recombination, the disrupted tk gene is  
substituted for the wild type viral tk+ gene in any  
recombinant virus. This serves as a marker for  
20 recombination since tk- human 143B cells infected with tk-  
virus are, in contrast to cells infected with wild type tk+  
virus, resistant to the toxic effect of BrdU. Recombinant  
virus can be scored by the viral pfu on 143B cells cultured  
25 in the presence of 125 mM BrdU.  
The frequency of recombinants derived in this fashion is of  
15 the order of 0.1% (Table 1).

Table 1: Generation of Recombinant Vaccinia Virus by  
Standard Homologous Recombination

Virus*	DNA	Titer without BrdU	Titer with BrdU	% Recombinant**
vaccinia ----		$4.6 \times 10^7$	$3.0 \times 10^3$	0.006
vaccinia 30ng pE/Lova		$3.7 \times 10^7$	$3.2 \times 10^4$	0.086
vaccinia 300ng pE/Lova		$2.7 \times 10^7$	$1.5 \times 10^4$	0.056

\* vaccinia virus strain vNot1

\*\* % Recombinant = (Titer with BrdU/Titer without  
BrdU)x100

This recombination frequency is too low to permit  
efficient construction of a cDNA library in a vaccinia  
vector. The following two procedures were used to generate  
an increased frequency of vaccinia virus recombinants.

(i) One factor limiting the frequency of viral  
recombinants generated by homologous recombination  
following transfection of a plasmid transfer vector into  
vaccinia virus infected cells is that viral infection is  
highly efficient whereas plasmid DNA transfection is  
relatively inefficient. As a result many infected cells do  
not take up recombinant plasmids and are, therefore,  
capable of producing only wild type virus. In order to  
reduce this dilution of recombinant efficiency, a mixture  
of naked viral DNA and recombinant plasmid DNA was  
transfected into Fowl Pox Virus (FPV) infected mammalian  
cells. As previously described by others (Scheifflinger,  
F., et al., 1992, Proc. Natl. Acad. Sci. USA 89:9977-9981),  
FPV does not replicate in mammalian cells but provides  
necessary helper functions required for packaging mature



vaccinia virus particles in cells transfected with non-infectious naked vaccinia DNA. This modification of the homologous recombination technique alone increased the frequency of viral recombinants approximately 35 fold to 3.5% (Table 2).

Table 2: Generation of Recombinant Vaccinia Virus by Modified Homologous Recombination

Virus	DNA	Titer		
		without BrdU	with BrdU	% Recombinant*
FPV	None	0	0	0
None	vaccinia WR	0	0	0
FPV	vaccinia WR	$8.9 \times 10^6$	$2.0 \times 10^2$	0.002
FPV	vaccinia WR + pE/Lova (1:1)	$5.3 \times 10^6$	$1.2 \times 10^5$	2.264
FPV	vaccinia WR + pE/Lova (1:10)	$8.4 \times 10^5$	$3.0 \times 10^4$	3.571

Table 2: Confluent monolayers of BSC1 cells ( $5 \times 10^5$  cells/well) were infected with moi=1.0 of fowlpox virus strain HP1. Two hours later supernatant was removed, cells were washed 2X with Opti-Mem I media, and transfected using lipofectamine with 600ng vaccinia strain WR genomic DNA either alone, or with 1:1 or 1:10 (vaccinia:plasmid) molar ratios of plasmid pE/Lova. This plasmid contains a fragment of the ovalbumin cDNA, which encodes the SIINFEKL epitope, known to bind with high affinity to the mouse class I MHC molecule K<sup>b</sup>. Expression of this minigene is controlled by a strong, synthetic Early/Late vaccinia promoter. This insert is flanked by vaccinia tk DNA. Three days later cells were harvested, and virus extracted by three cycles

of freeze/thaw in dry ice isopropanol/ 37°C water bath.  
Crude virus stocks were titered by plaque assay on human  
TK- 143B cells with and without BrdU.

\* %Recombinant = (Titer with BrdU/Titer without BrdU)x  
100

5

(ii) A further significant increase in the  
frequency of viral recombinants was obtained by  
transfection of FPV infected cells with a mixture of  
recombinant plasmids and the two large approximately 80  
kilobases and 100 kilobases fragments of vaccinia virus  
v7.5/tk DNA produced by digestion with Not I and Apa I  
restriction endonucleases. Because the Not I and Apa I  
sites have been introduced into the tk gene, each of these  
large vaccinia DNA arms includes a fragment of the tk gene.  
Since there is no homology between the two tk gene  
fragments, the only way the two vaccinia arms can be linked  
is by bridging through the homologous tk sequences that  
flank the inserts in the recombinant transfer plasmid. The  
results in Table 3 show that >99% of infectious vaccinia  
virus produced in triply transfected cells is recombinant  
for a DNA insert as determined by BrdU resistance of  
infected tk- cells.

Table 3: Generation of 100% Recombinant Vaccinia Virus  
using Tri-Molecular Recombination

Virus	DNA	Titer without BrdU	Titer with BrdU	% Recombinant*
FPV	Uncut	$2.5 \times 10^6$	$6.0 \times 10^3$	0.24
	v7.5/tk			
FPV	NotI/ApaI	$2.0 \times 10^6$	0	0
	v7.5/tk arms			
FPV	NotI/ApaI	$6.8 \times 10^6$	$7.4 \times 10^4$	100
	v7.5/tk arms + 1:1 pE/Lova			

- 47 -

Table 3: Genomic DNA from vaccinia strain V7.5/tk (1.2 micrograms) was digested with ApaI and NotI restriction endonucleases. The digested DNA was divided in half. One of the pools was mixed with a 1:1 (vaccinia:plasmid) molar ratio of pE/Lova. This plasmid contains a fragment of the ovalbumin cDNA, which encodes the SIINFEKL epitope, known to bind with high affinity to the mouse class I MHC molecule K<sup>b</sup>. Expression of this minigene is controlled by a strong, synthetic Early/Late vaccinia promoter. This insert is flanked by vaccinia tk DNA. DNA was transfected using lipofectamine into confluent monolayers (5 X 10<sup>5</sup> cells/well) of BSC1 cells, which had been infected 2 hours previously with moi=1.0 FPV. One sample was transfected with 600ng untreated genomic V7.5/tk DNA. Three days later cells were harvested, and the virus was extracted by three cycles of freeze/thaw in dry ice isopropanol/ 37° C water bath. Crude viral stocks were plaqued on TK- 143 B cells with and without BrdU selection.

\* %Recombinant = (Titer with BrdU/Titer without BrdU)x 100

### 6.3. CONSTRUCTION OF A REPRESENTATIVE cDNA LIBRARY IN VACCINIA VIRUS

A cDNA library is constructed in the vaccinia vector to demonstrate representative expression of known cellular mRNA sequences.

Additional modifications have been introduced into the p7.5/tk transfer plasmid and v7.5/tk viral vector to enhance the efficiency of recombinant expression in infected cells. These include introduction of translation initiation sites in three different reading frames and of both translational and transcriptional stop signals as well as additional restriction sites for DNA insertion.

5 First, the HindIII J fragment (vaccinia tk gene) of p7.5/tk was subcloned from this plasmid into the HindIII site of pBS phagemid (Stratagene) creating pBS.Vtk.

10 Second, a portion of the original multiple cloning site of pBS.Vtk was removed by digesting the  
5 plasmid with SmaI and PstI, treating with Mung Bean Nuclease, and ligating back to itself, generating  
15 pBS.Vtk.MCS-. This treatment removed the unique SmaI, BamHI, SalI, and PstI sites from pBS.Vtk.

Third, the object at this point was to introduce  
a new multiple cloning site downstream of the 7.5k promoter  
10 in pBS.Vtk.MCS-. The new multiple cloning site was  
20 generated by PCR using 4 different upstream primers, and a common downstream primer. Together, these 4 PCR products would contain either no ATG start codon, or an ATG start codon in each of the three possible reading frames. In  
25 addition, each PCR product contains at its 3 prime end,  
15 translation stop codons in all three reading frames, and a vaccinia virus transcription double stop signal. These 4  
30 PCR products were ligated separately into the NotI/ ApaI sites of pBS.Vtk.MCS-, generating the 4 vectors,  
p7.5/ATG0/tk, p7.5/ATG1/tk, p7.5/ATG3/tk, and p7.5/ATG4/tk whose sequence modifications relative to the p7.5/tk vector  
20 are shown in Figure 2. Each vector includes unique BamHI, SmaI, PstI, and SalI sites for cloning DNA inserts that  
35 employ either their own endogenous translation initiation site (in vector p7.5/ATG0/tk) or make use of a vector translation initiation site in any one of the three  
40 possible reading frames (p7.5/ATG1/tk, p7.5/ATG3/tk, and  
25 p7.5/ATG4/tk).

In a model experiment cDNA was synthesized from  
poly-A+ mRNA of a murine tumor cell line (BCA39) and  
45 ligated into each of the four modified p7.5/tk transfer plasmids. Twenty micrograms of Not I and Apa I digested  
v/tk vaccinia virus DNA arms an equal was transfected  
30 together with an equimolar mixture of the four recombinant  
50 plasmid cDNA libraries into FPV helper virus infected BSC-1

5 cells for tri-molecular recombination. The virus harvested had a total titer of  $6 \times 10^6$  pfu of which greater than 90% were BrdU resistant.

10 In order to characterize the size distribution of cdna inserts in the recombinant vaccinia library,  
5 individual isolated plaques were picked using a sterile pasteur pipette and transferred to 1.5ml tubes containing 100  $\mu$ l Phosphate Buffered Saline (PBS). Virus was released  
15 from the cells by three cycles of freeze/thaw in dry ice/isopropanol and in a 37° C water bath. Approximately one third of each virus plaque was used to infect one well  
10 of a 12 well plate containing tk- human 143B cells in 250  $\mu$ l final volume . At the end of the two hour infection period each well was overlaid with 1 ml DMEM with 2.5% fetal bovine serum (DMEM-2.5) and with BUdR sufficient to bring the final concentration to 125  $\mu$ g/ml. Cells were  
25 incubated in a CO<sub>2</sub> incubator at 37°C for three days. On the third day the cells were harvested, pelleted by centrifugation, and resuspended in 500  $\mu$ l PBS. Virus was released from the cells by three cycles of freeze/ thaw as described above. Twenty percent of each virus stock was used to infect a confluent monolayer of BSC-1 cells in a 50mm tissue culture dish in a final volume of 3 ml DMEM-  
30 2.5. At the end of the two hour infection period the cells were overlaid with 3 ml of DMEM-2.5. Cells were incubated in a CO<sub>2</sub> incubator at 37°C for three days. On the third day the cells were harvested, pelleted by centrifugation, and resuspended in 300  $\mu$ l PBS. Virus was released from the cells by three cycles of freeze/ thaw as described above.  
40 25 One hundred microliters of crude virus stock was transferred to a 1.5 ml tube, an equal volume of melted 2% low melting point agarose was added, and the virus/agarose mixture was transferred into a pulsed field gel sample block. When the agar worms were solidified they were removed from the sample block and cut into three equal  
45 30 sections. All three sections were transferred to the same 1.5 ml tube, and 250 $\mu$ l of 0.5M EDTA, 1% Sarkosyl, 0.5mg/ml

5 Proteinase K was added. The worms were incubated in this  
solution at 37°C for 24 hours. The worms were washed  
several times in 500µl 0.5X TBE buffer, and one section of  
10 each worm was transferred to a well of a 1% low melting  
point agarose gel. After the worms were added the wells  
5 were sealed by adding additional melted 1% low melting  
point agarose. This gel was then electrophoresed in a  
Bio-Rad pulsed field gel electrophoresis apparatus at  
15 200volts, 8 second pulse times, in 0.5X TBE for 16 hours.  
The gel was stained in ethidium bromide, and portions of  
agarose containing vaccinia genomic DNA were excised from  
20 the gel and transferred to a 1.5 ml tube. Vaccinia DNA was  
purified from the agarose using β-Agarase (Gibco) following  
the recommendations of the manufacturer. Purified vaccinia  
DNA was resuspended in 50 µl ddH<sub>2</sub>O. One microliter of each  
DNA stock was used as the template for a Polymerase Chain  
25 Reaction (PCR) using vaccinia TK specific primers MM428 and  
15 MM430 (which flank the site of insertion) and KlenTaq  
Polymerase (Clontech) following the recommendations of the  
manufacturer in a 20µl final volume. Reaction conditions  
30 included an initial denaturation step at 95°C for 5 minutes,  
followed by 30 cycles of: 94°C 30 seconds, 55°C 30 seconds,  
68°C 3 minutes. Two and a half microliters of each PCR  
20 reaction was resolved on a 1% agarose gel, and stained with  
ethidium bromide. Amplified fragments of diverse sizes  
35 were observed. When corrected for flanking vector  
sequences amplified in PCR the inserts range in size  
between 300 and 2500 bp.

40 The vaccinia virus cDNA library was further  
25 characterized in terms of the representation of clones  
homologous to the murine alpha tubulin sequence. Twenty  
separate pools with an average of either 300, 900 or 2,700  
45 viral pfu from the library were amplified by infecting a  
monolayer of 143B tk- cells in the presence of BrdU. DNA  
was extracted from each infected culture after three days  
30 and assayed for the presence of an alpha tubulin sequence  
by PCR with tubulin specific primers. Poisson analysis of  
50

5 the frequency of positive pools indicates a frequency of  
one alpha tubulin recombinant for every 2000 to 3000 viral  
pfu. This is not significantly different from the expected  
10 frequency of alpha tubulin sequences in this murine tumor  
cell line and suggests representative expression of this  
5 randomly selected sequence in the vaccinia cDNA library.

#### 6.4. DISCUSSION

15 The above-described tri-molecular recombination  
strategy yields close to 100% viral recombinants. This is  
a highly significant improvement over current methods for  
10 generating viral recombinants by transfection of a plasmid  
transfer vector into vaccinia virus infected cells. This  
20 latter procedure yields viral recombinants at a frequency  
of the order of only 0.1%. The high yield of viral  
recombinants in tri-molecular recombination makes it  
25 possible, for the first time, to efficiently construct  
15 genomic or cDNA libraries in a vaccinia virus derived  
vector. In the first series of experiments a titer of  $6 \times 10^6$   
30 recombinant virus was obtained following transfection  
with a mix of 20 micrograms of Not I and Apa I digested  
vaccinia vector arms together with an equimolar  
concentration of tumor cell cDNA. This technological  
20 advance creates the possibility of new and efficient  
35 screening and selection strategies for isolation of  
specific genomic and cDNA clones.

The tri-molecular recombination method as herein  
disclosed may be used with other viruses such as mammalian  
40 viruses including vaccinia and herpes viruses. Typically,  
25 two viral arms which have no homology are produced. The  
only way that the viral arms can be linked is by bridging  
through homologous sequences that flank the insert in a  
45 transfer vector such as a plasmid. When the two viral arms  
and the transfer vector are present in the same cell the  
only infectious virus produced is recombinant for a DNA  
30 insert in the transfer vector.

5 Libraries constructed in vaccinia and other  
mammalian viruses by the tri-molecular recombination method  
of the present invention may have similar advantages to  
10 those described here for vaccinia virus and its use in  
identifying target antigens in the CTL screening system of  
5 the invention. Similar advantages are expected for DNA  
libraries constructed in vaccinia or other mammalian  
viruses when carrying out more complex assays in eukaryotic  
15 cells. Such assays include but are not limited to  
screening for DNA encoding receptors and ligands of  
eukaryotic cells.

20 7. EXAMPLE: INDUCTION OF CYTOTOXIC T CELLS  
SPECIFIC FOR HUMAN TUMORS IN  
HLA AND HUMAN CD8 TRANSGENIC MICE

25 In this example, HLA and human CD8 transgenic  
mice were tolerized with a non-tumorigenic, immortalized  
15 normal human cell line that does not express costimulator  
activity for murine T cells and were subsequently immunized  
with B7 (costimulator) transfected tumor cells derived by  
30 *in vitro* mutagenesis or oncogene transformation from that  
same normal cell line. The HLA transgene permits selection  
of a high affinity, HLA- restricted T cell repertoire in  
20 the mouse thymus. In addition, a human CD8 transgene is  
required because murine CD8 does not interact efficiently  
35 with human class I MHC. Subsequent to immunization with B7  
transfected tumor cells, splenic CD8+ T cells are isolated  
and stimulated again *in vitro* in the absence of  
40 costimulation with non-tumorigenic, immortalized human  
25 cells. Two pathways of tolerance induction for antigens  
shared by the tumorigenic and non-tumorigenic cell lines  
may be activated through these manipulations. As known to  
45 those skilled in the art, antigen exposure in very young  
mice favors tolerance induction by mechanisms that may  
30 include both clonal deletion and induction of T cell  
anergy. Further, restimulation of activated T cells



5 through their antigen-specific receptors in the absence of  
costimulator activity induces apoptotic elimination of  
those T cells. This immunization regimen enriched for  
10 tumor-specific CTL that did not crossreact with the  
homologous normal cells.

5 A series of tumor cell lines were used that were  
all derived from a single immortalized, non-tumorigenic  
15 cell line. The non-tumorigenic cells were used to induce  
tolerance to the large number of normal human proteins that  
are also expressed in tumor cells. Availability of a panel  
of tumors independently derived from the same normal cells  
10 by diverse carcinogens or oncogene transformation makes it  
possible to filter out antigenic changes that are  
carcinogen specific or that may arise by random genetic  
drift during *in vitro* propagation of a tumor cell line.

25 Cytotoxic T cells specific for human bladder  
tumor cell lines were induced and isolated from (HLA-A2/K<sup>b</sup> x  
15 human CD8)F<sub>1</sub> hybrid double transgenic mice that had been  
tolerized to the normal cell line from which the tumors  
derive. Neonatal mice were injected intraperitoneally with  
30 5 x 10<sup>6</sup> non-tumorigenic SV-HUC. Seven weeks later they were  
immunized with 5 x 10<sup>6</sup> B7.1 transfected ppT11.B7 tumor  
cells. ppT11 is one of several independent tumor cell  
20 lines derived from SV-HUC by *in vitro* carcinogenesis  
(Christian, et al., 1987, Cancer Res. 47: 6066-6073; Pratt,  
35 et al., 1992, Cancer Res. 52: 688-695; Bookland, et al.,  
1992, Cancer Res. 52: 1606-1614). One week after  
immunization, spleen was removed and a single cell  
40 suspension prepared. CD8 positive T cell precursors were  
25 enriched on anti-Lyt-2 coated MACS (Magnetic cell sorting  
beads) as recommended by the manufacturer (Miltenyi  
Biotech, Sunnyvale, CA). 1.5 x 10<sup>6</sup> CD8 enriched T cells  
45 were then restimulated *in vitro* with 4 x 10<sup>5</sup> SV-HUC in 3 ml  
of RPMI 1640 + 10% fetal bovine serum. The rationale is  
30 that any SV-HUC specific T cells that escape neonatal  
tolerance induction and are activated *in vivo* by

5 stimulation with crossreactive determinants of ppT11.B7,  
might now be induced to undergo apoptosis by restimulation  
in vitro with costimulator activity negative SV-HUC cells.  
10 After 24 hours, T cells are again stimulated with ppT11.B7  
in the presence of 2000 Units/ml of recombinant murine IL-  
5 6. On day 7 the cycle of SV-HUC stimulation followed 24  
hours later by restimulation with ppT11.B7 is repeated.  
15 This second round of stimulation with ppT11.B7 is carried  
out in the presence of 10 nanogram/ml recombinant murine  
IL-7 and 50 Units/ml recombinant murine IL-2. CTL activity  
is determined 5 days later by standard chromium release  
20 10 assay from labeled targets SV-HUC, ppT11.B7 and YAC-1, a  
cell line sensitive to non-specific killing by murine NK  
cells. The results in Table 4 show that CTL from ppT11.B7  
immunized mice that were not previously tolerized to SV-HUC  
25 are equally reactive with SV-HUC and ppT11 target cells.  
In contrast, following neonatal tolerization with SV-HUC,  
15 cytolytic T cells at an effector:target ratio of 5:1 are  
significantly more reactive with ppT11.B7 tumor cells than  
with SV-HUC. Note that B7 costimulator activity is not  
30 required at the effector stage as similar results are  
obtained with B7 transfected or non-transfected target  
cells.  
20

Table 4: Tumor-specific response in (HLA-A2/K<sup>b</sup> x human CD8)F<sub>1</sub> hybrid transgenic mice neonatally tolerized with SV-HUC parental cells and then immunized with B7 costimulator transfected ppT11.B7 human bladder tumor cells.

Target	Tolerogen: Immunogen:	Effector:Target ratio			
		5:1	10:1	2:1	5:1
SV-HUC	None ppT11.B7	29	68	14	19
ppT11.B7		14	70	17	51
YAC-1		6	6	nd	3

nd = not done

The significance of this experimental protocol is that it offers a means of selecting murine, HLA-restricted cytolytic T cells specific for human epithelial tumor cells. As noted previously, it has proved exceedingly difficult to isolate such T cells directly from either patient PBL or tumor infiltrating lymphocytes of tumors other than melanoma and perhaps renal cell carcinoma. In addition, as emphasized in section 5.1.1, this same strategy can be implemented in two stages. Differentially immunogenic molecules of the human tumor can first be identified employing tumor-specific CTL restricted to a variety of different animal MHC. These antigens can, as described in Example 12, subsequently be characterized in human subjects or transgenic mice for the ability to be processed and presented in association with different human HLA types. An advantage of this two stage approach is that

5 numerous different MHC molecules are available in a variety  
of inbred strains and these can be employed to capture an  
10 equally broad range of tumor-specific immunogenic peptides  
in the initial screening.

5 8. EXAMPLE: HIGH-THROUGHPUT STRATEGY  
FOR SELECTION OF DNA RECOMBINANTS  
15 FROM A LIBRARY THAT ENCODES THE  
TARGET EPITOPES OF SPECIFIC  
CYTOTOXIC T CELLS

20 In this example, a model system was assayed to  
determine the level of enrichment that can be obtained  
through a procedure that selects for DNA recombinants that  
encode the target epitopes of tumor specific cytotoxic T  
cells.

25 8.1. METHODS AND RESULTS

15 A specific vaccinia recombinant that encodes a  
well characterized ovalbumin peptide (SIINFEKL) (SEQ ID  
NO:φφ) was diluted with non-recombinant virus so that it  
30 constituted either 0.2%, 0.01%, or 0.001% of viral pfu.  
This ovalbumin peptide is known to be processed and  
presented to specific CTL in association with the murine  
35 class I MHC molecule H-2K<sup>b</sup>. An adherent monolayer of MC57G  
cells that express H-2K<sup>b</sup> were infected with this viral mix  
at m.o.i.=1 (approximately 5 x 10<sup>5</sup> cell/well). MC57G cells  
do not themselves express ovalbumin peptide, but do express  
40 H-2K<sup>b</sup>, which allows them to associate with and present  
ovalbumin peptide to the T cells.

25 Following 12 hours of infection with the  
recombinant vaccinia virus expressing ovalbumin peptide,  
ovalbumin peptide-specific CTL, derived by repeated in  
45 vitro stimulation of ovalbumin primed splenic T cells with  
the immunodominant ovalbumin SIINFEKL peptide, were added  
for 30 min.

30

During this time, some of the adherent cells infected with a recombinant particle that leads to expression of the ovalbumin peptide interacted with a specific cytotoxic T cell and underwent a lytic event. Cells that underwent a lytic event were released from the monolayer. After 30 min, the monolayer was gently washed, and the floating cells and the remaining adherent cells were separately harvested.

Virus extracted from each cell population was titred for the frequency of ovalbumin recombinant viral pfu. Virus extracted from floating cells was then used as input to another enrichment cycle with fresh adherent MC57G cells and ovalbumin peptide-specific CTL. It was observed that, following enrichment of VVova to greater than 10% of total virus, further enrichment of the recombinant virus was accelerated if the m.o.i. in succeeding cycles was reduced from 1 to 0.1. The results, presented in Table 5, demonstrate marked enrichment of VVova recombinant virus from an initial concentration of 0.2% to 49% or from 0.01% to 39% in 5 enrichment cycles and from 0.001% to 18% in 6 enrichment cycles. Note that with  $5 \times 10^5$  adherent MC57G cells per well and m.o.i = 1, an initial concentration of 0.001% VVova recombinant virus is equivalent, on average, to seeding only 5 recombinant pfu among  $5 \times 10^5$  wild type vaccinia virus in a single culture well. A very substantial enrichment is achieved even under these conditions.

Table 5: Multiple Cycles of Enrichment for Vvova

Enrichment cycle #	% VVova in Floating cells*		
	Exp. 1	Exp. 2	Exp. 3
moi = 1    0	0.2	0.01	0.001
1	2.1	0.3	nd

- 58 -

5		2	4.7	1.1	nd
		3	9.1	4.9	nd
		4	14.3	17.9	1.4
10		5	24.6		3.3
		6			18.6

5

moi=0.1    5                    48.8                    39.3

15

\*    % Vvova = (Titer with BrdU / Titer without BrdU) x 100  
      nd = not determined

10

## 8.2. DISCUSSION

20

The above-described selection method for isolating DNA clones that encode target epitopes of specific cytotoxic T cells from a viral library is far more efficient than existing methods for accomplishing this same goal. Prior to the present invention, the most widely employed method requires transfection of numerous small pools of recombinant plasmids into separate target populations in order to assay T cell stimulation by a minor component of some pool. Because this requires screening out many negative plasmid pools, it is a far more labor intensive procedure than the positive selection method described herein. For a given investment of resources, the method described here can detect positive DNA clones that occur at a much lower frequency than would otherwise be possible. The design principle of this strategy can be directly extended to screening and selection of DNA clones with specific antibodies as well as with CTL.

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### 9. EXAMPLE: IDENTIFICATION OF POTENTIAL TUMOR-SPECIFIC ANTIGENS THAT ARE DIFFERENTIALLY EXPRESSED IN TUMORS

45

Identification of genes that are differentially expressed in human tumors, cancers, or infected cells could facilitate development of broadly effective human vaccines. Most methods for identification of differential gene

50

55

5 expression are variations of either subtractive hybridization or the more recently described differential display technique.

10 Representational difference analysis (RDA) is a subtractive hybridization based method applied to  
5 "representations" of total cellular DNA (Lisitsyn, N. and N., M. Wigler. 1993. Cloning the differences between two complex genomes. Science 259: 946-951). The  
15 differential display methods of Liang and Pardee (1992, Science 257:967-971) employ an arbitrary 10 nucleotide primer and anchored oligo-dT to PCR amplify an arbitrary  
10 subset of fragments from a more complex set of DNA molecules. As described below (Section 9.2), we have modified differential display to enhance the efficiency with which differentially expressed genes can be identified. In this example we illustrate how application  
20 of these methods to a related set of tumors independently  
25 derived from a single non-tumorigenic, immortalized cell line facilitates identification of tumor-specific gene products.

30 Experiments described by Sahasrabudhe, et al., (1993, J. Immunology 151:6302-6310), focused on a set of murine tumor cell lines, all of which were independently  
20 derived from a single cloned, non-tumorigenic BALB/c embryonic fibroblast cell line. These tumors were of  
35 particular interest because they are known to share an immunoprotective antigen. The goal of these experiments was to arrive at a molecular definition of that shared tumor antigen. The ready availability of tumor cells, as  
40 well as the normal cells from which they were derived, was  
25 exploited for efficient analysis of differential gene expression and tumor immunogenicity by the methods described below.

45 The availability of multiple tumors independently derived from the same normal cells by diverse carcinogens  
30 (or oncogene transformation) also makes it possible to filter out antigenic changes that are carcinogen specific

5 or that may arise as a result of random genetic drift  
during *in vitro* propagation of tumor cells. (See Example  
10, where a series of human tumor cell lines is described  
that satisfy the requirements of this analysis).

5 The relationship between the process of  
transformation and expression of shared tumor rejection  
antigens was investigated by characterizing the  
15 immunological relationships among a series of murine tumors  
(BCA 34, BCA 39, BCA 22, and BCB 13) independently derived  
from B/C-N7.1C.1, a contact inhibited, non-tumorigenic  
clone of a continuous fibroblast cell line derived from a  
20 BALB/c fetus (Collins, et al., 1982, Nature 299: 167; Lin,  
et al., 1985, JNCI 74: 1025). Although the proximal cause  
of tumor transformation may have been a carcinogen induced  
mutation, this model afforded the opportunity to determine  
if the process of transformation is also associated with  
25 expression of a limited number of shared antigens.

15 As reported by Sahasrabudhe, et al. (1993, J.  
Immunology 151: 6302-6310), immunological analysis  
demonstrates that three of four B/c.N derived tumors confer  
30 crossprotective immunity against each other. Concordant  
with the *in vivo* cross-protection data, cytolytic T cell  
clones from mice immunized with one of the immunologically  
20 related tumors specifically lyse all three immunologically  
related tumors but, importantly, do not react with the  
35 parental B/c.N cells or with the immunologically  
independent BCB 13 tumor. The observation of immunological  
cross-reactivity among a group of tumors independently  
40 derived from a cloned non-tumorigenic parental cell line  
25 strongly suggests that a non-random transformation  
associated process gives rise to recurrent expression of  
the same tumor antigen(s). Two methods for analyzing  
45 differential gene expression, representational difference  
analysis (RDA) and modified differential display, were  
employed to isolate cDNA that might encode the relevant  
30 tumor antigen(s).



### 9.1. REPRESENTATIONAL DIFFERENCE ANALYSIS (RDA)

The PCR SELECT™ variation of RDA is marketed by Clontech (Palo Alto, CA). The following general protocol outlined in the text and in Fig. 3 is a summary of the manufacturer's recommendations. cDNA is synthesized from both a tracer (represented by BCA 39 tumor mRNA) and a driver (represented by parental B/c.N mRNA). "Representations" of both tracer and driver cDNA are created by digestion with RsaI which cuts the four-base recognition sequence GTAC to yield blunt end fragments. Adaptors, which eventually serve as primer sites for PCR, are ligated to the 5' ends of only the tracer cDNA fragments (Fig. 3). Two aliquots of tracer representation are separately ligated with two different adaptors. A series of two hybridizations are carried out. In the first set of hybridizations, each adaptor ligated tracer sample is denatured and hybridized with a ten fold excess of the denatured representation of driver cDNA for 8 hours. Under these conditions re-annealing of all molecules is incomplete and some of both the high and low copy molecules remain single stranded. Since re-annealing rates are faster for more abundant species, this leads to normalization of the distribution through relative enrichment of low copy number single stranded molecules. The two hybridization reactions with each of the different adaptor ligated tracer cDNA representations are then combined *without fractionation or further denaturation* but with addition of more freshly denatured driver in a second hybridization reaction that is allowed to proceed further to completion, approximately 20 hours.

An aliquot of the products from the second hybridization is used as a template for a high stringency PCR reaction, using the known sequences at the 5' ends of the ligated adaptors as primers. The key here is that only tumor tracer sequences that 1) remain single stranded through the first hybridization and 2) hybridize to a

5 complementary tracer sequence ligated to the alternate  
adapter in the second hybridization can be exponentially  
amplified during PCR. This excludes both tracer and driver  
10 species that either remain single stranded or that have  
hybridized to excess driver (since they have a  
5 complementary primer at only one or neither end of the  
molecule), as well as tracer sequences that hybridize to a  
molecule with the same adapter (because the adapters are  
15 longer than the primers and hybridize to their own  
complement with higher affinity when it is present on the  
opposite end of a denatured single stranded molecule - a  
20 reaction termed "Suppression PCR" by Clontech). Finally, a  
second high stringency PCR is performed using nested  
primers built into the adapters so as to further reduce  
background and enrich for differentially expressed  
25 sequences. The products of the second PCR are  
electrophoresed and visualized on an agarose gel.  
15 Individual bands are excised and subcloned for further  
analysis.

30 **9.2. REPRESENTATIONAL DIFFERENCE  
ANALYSIS OF GENES THAT ENCODE  
POTENTIAL TUMOR IMMUNOGENS**

20 This example describes how the PCR SELECT™ cDNA  
35 subtraction method (Clontech Laboratories) was successfully  
employed to identify a strong candidate for the shared  
tumor antigen in a set of immunologically related murine  
tumors.

40 As shown in Fig. 4, subtraction of a fragmented  
25 representation of normal cell cDNA from a similar  
representation of BCA 39 tumor cDNA resulted in  
identification of a series of seven clearly distinguishable  
45 subtraction products ranging in size from approximately 300  
to 2200 base pairs. To confirm that these DNA fragments  
30 were indeed differentially expressed, each band was cloned  
into Bluescript plasmid (Stratagene) and the DNA inserts of  
50

5 at least 5 colonies from each band were analyzed by  
Northern blot hybridization to RNA of the five different  
cell lines: the parental cells, the three immunologically  
10 crossreactive and the one non-crossreactive tumor cell  
line. Representative results for clone 3f derived from RDA  
5 band 1 are shown in Fig. 5A.

The probe hybridized to at least three  
15 transcripts in the BCA 22, 34 and 39 tumor mRNA.  
Expression of these transcripts is unique to these three  
immunologically crossreactive tumors. Minimal hybridization  
is detected with RNA of the parental B/c.N cells or of the  
10 non-crossreactive BCB 13 tumor. Similar results were  
20 obtained in four Northern blots with independent RNA  
preparations. The integrity and relative loading of RNA  
samples was determined by hybridization to a fragment of  
the mouse G3PDH gene (Fig. 5B).

The sequence of clone 3f was determined and found  
15 to be strongly homologous to a portion of the sequence of a  
murine intracisternal type A particle (IAP element) (Aota,  
et al., 1987, Gene 56: 1-12). IAPs are endogenous  
30 retrovirus-like particles that localize to the cisternae of  
the endoplasmic reticulum. They are non-infectious because  
they do not encode functional packaging proteins; the  
20 potential env region of the sequence contains many  
35 conserved stop codons (Kuff and Lueders, 1988, Advances in  
Cancer Research 51:183-276). Most IAPs do encode a 73 kDa  
major gag protein, and a pol polypeptide with some reverse  
40 transcriptase properties (Wilson and Kuff, 1972, Proc.  
Natl. Acad. Sci. USA 69: 1531-1536). Expression of IAP  
25 transcripts has been described in various mouse primary  
tumors (including plasmacytomas, papillomas, carcinomas,  
mammary tumors, sarcomas, hepatomas) and established mouse  
45 tumors and cell lines (including Friend erythroleukemias,  
myelomonocytic leukemia, T lymphomas, myelomas). Although  
30 expression in normal thymus may be elevated, only very low  
levels of expression are detected in most normal mouse

somatic tissues (Kuff and Lueders, 1988, Advances in Cancer Research 51: 183-276).

### 9.3. CHARACTERIZATION OF DIFFERENTIALLY EXPRESSED GENE SEQUENCE FROM RDA

Semi-quantitative PCR is a more sensitive test for differential expression than Northern Blot analysis. Clone 3f sequence specific primers were used to amplify full length oligo- dT primed cDNA from both the BCA 39 tumor and the parental cell line. Amplification with mouse tubulin primers was used to normalize the amount of template between the two cell lines. Equal aliquots of each template were amplified through a variable number of PCR cycles. In each case an estimate of the relative template concentration was derived by fitting a line to the portion of the amplification curve in which product increases exponentially with cycle number. The assumption is that in this region yield is a linear function of (initial template concentration)\*(a<sup>n</sup>) where a = average amplification per cycle in that PCR region, usually between 1.5 and 1.8, and n = cycle number. It was determined that expression of the 3f fragment is at least 7 times greater in the BCA39 tumor cDNA relative to the parental B/c.N.

Differential expression in tumor RNA was confirmed for the inserts of 12 additional clones derived from the six other RDA bands. Northern analysis showed the identical hybridization pattern characteristic of IAP transcripts as observed for clone 3f. The sequence of each clone was determined and found to be homologous to other regions of the IAP genome. A map of the relative position of 10 unique RDA clones is shown in Fig. 6. It can be seen that cumulatively these inserts cover most of the IAP genome.

It is particularly striking that expression of these IAP sequences is shared among the three immunologically crossreactive tumors (BCA 39, BCA 34, and

5 BCA 22) but is absent or very low in both the B/c.N  
parental cells and the immunologically unrelated BCB 13  
tumor. An IAP epitope is, therefore, a strong candidate  
10 for this shared tumor antigen. Experiments are in progress  
to transfect the different RDA clones into antigen negative  
5 B/c.N cells which will then be tested for sensitization to  
lysis by tumor-specific CTL. Transcriptional activation of  
endogenous retroviral elements including IAP may represent  
15 a new class of shared tumor rejection antigens. It has  
been reported (de Bergeyck, et al., 1994, Eur. J. Immunol.  
24:2203-2212) that the tumor antigen LEC-A on the murine  
10 LEC spontaneous leukemia is also encoded by the gag gene of  
an IAP element. Recently, a tumor rejection antigen of a  
murine colon tumor, CT26, was found to be encoded by  
another type of endogenous retrovirus, a type C particle  
25 (Huang, et al., 1996, Proc. Natl. Acad. Sci. USA  
93:9730-9735). Retroviral-like elements are also present  
15 in the human genome: expression of the pol gene has been  
detected in human breast (Moyret, et al., 1988, Anticancer  
Res. 8:1279-1283) and colorectal carcinomas (Moshier, et  
30 al., 1986, Biochem. Biophys. Res. Commun. 139:1071-1077),  
and antibody to the gag gene product has been reported in  
20 the sera of patients with human seminoma (Sauter, et al.,  
1995, J. Virol. 69:414-421) and renal cell carcinoma  
35 (Wahlstrom, et al., 1985, Lab. Invest. 53:464-469).

#### 9.4. MODIFIED DIFFERENTIAL DISPLAY OF GENES ENCODING POTENTIAL TUMOR IMMUNOGENS

40  
25 In the following example, the differential  
display methods of Liang and Pardee (1992, Science  
257:967-971) were modified to improve resolution of DNA  
45 fragments and reduce the frequency of false positives.

The differential display method as originally  
described by Liang and Pardee (1992, Science 257:967-971)  
30 employs an arbitrary 10 nucleotide primer and anchored  
oligo-dT to PCR amplify an arbitrary subset of fragments  
50

5 from a more complex set of DNA molecules. In principle,  
differences among the fragments generated from normal and  
tumor cell lines should reflect differences in gene  
10 expression in the two cell types. In practice, this method  
sometimes works well but often gives rise to numerous false  
5 positives. That is, bands which appear to be  
differentially displayed are, upon further  
15 characterization, found not to be differentially expressed.  
This is presumably due to variable PCR amplification of  
individual species in complex populations and a relatively  
high background that can obscure less prominent bands.  
20 Since considerable effort is required to establish  
differential expression, these endemic false positives are  
costly in terms of efficiency and productivity.

A single arbitrary primer may also be used for  
differential display, as described by Welch et al. (3,4).  
25 Use of single primers does, however, require synthesis of a  
15 much larger set of independent primers to achieve the same  
coverage of a complex cDNA population.

Hence, there exists a need for improved  
30 differential display methods that improve resolution of DNA  
fragments and that reduce the frequency of false positives.

In order to improve the resolution of fragments  
20 and reduce the frequency of false positives, a second  
35 arbitrary primer was substituted for the anchored oligo-dT  
employed in PCR amplification. This results in fewer DNA  
products in each PCR reaction so that individual DNA  
fragments can be more reliably resolved on sequencing gels.

40  
25 Because each subset of fragments generated in  
this modified differential display protocol is a smaller  
representation of total cDNA, more primer pairs are  
45 required for adequate sampling. Employing the negative  
binomial distribution, it can be predicted that if 12  
independent primers are utilized in all 66 possible primer  
30 pair combinations there is a greater than 85% probability  
50 that for an average size eukaryotic cDNA at least one

primer pair will amplify a representative PCR fragment of size  $\geq 70$ bp.

Table 6 lists the sequences of the 12 arbitrary decamers from which primer pairs are selected for modified differential display. The specific primers were chosen on the basis of their sequence diversity, 3' hybridization affinity, and minimal pair-wise hybridization.

---

TABLE 6: ARBITRARY PRIMERS FOR MODIFIED DIFFERENTIAL DISPLAY

---

10	20	TAC AAC GAG G MR_1	TCG GTC ACA G MR_9
		GTC AGA GCA T MR_2	ATC TGG TAG A MR_10
		GGA CCA AGT C MR_5	CTT ATC CAC G MR_11
		TCA GAC TTC A MR_7	CAT GTC TCA A MR_12
	25	TAC CTA TGG C MR_8	GAT CAA GTC T MR_14
	15	TGT CAC ATA C MR_15	CTG ATC CAT G Ldd1

---

A separate cDNA synthesis reaction with 0.1  $\mu$ g polyA-RNA and Superscript II Reverse Transcriptase (Gibco/BRL) is carried out with each primer. Five percent of the cDNA product made with each member of a primer pair is mixed together with that primer pair for amplification in 30 PCR cycles using Klen Taq Polymerase Mix (Clontech). The PCR primers are used for cDNA synthesis to avoid the 3' bias imposed by oligo-dT primed cDNA synthesis. The relative orientation of the two primers in cDNA is randomized by carrying out a separate synthesis with each primer. These cDNA can be mixed in the same combinations as the primers chosen for PCR amplification. PCR amplified cDNA fragments are resolved on 6% acrylamide gels and dried for autoradiography. Those bands which are differentially displayed in at least 2 tumor samples and not in the parental cells are cut out and rehydrated. An aliquot (1/5) of the DNA recovered is reamplified using the same

5 primer set and the same PCR conditions but without addition  
of isotope. This second PCR product is resolved on 1%  
10 agarose and individual bands are recovered by incubation  
with  $\beta$  agarase I (Gibco/BRL). Each DNA fragment recovered  
5 is cloned by blunt end ligation into the pcDNA3.1/Zeo (+)  
phagemid vector (Invitrogen). Since it is possible that a  
single band may include more than one molecular species, at  
15 least 4 different transformants with an insert of  
appropriate size are picked for further characterization.  
Northern analysis, RNase protection assays and  
semi-quantitative PCR are employed to confirm differential  
10 expression.

20 In murine tumor cell lines, it was observed that  
many more differentially expressed gene fragments appear to  
be identified by differential display than by RDA. In  
25 addition, RDA fragments give positive results on Northern  
blots exposed for only a few hours. In contrast, fragments  
15 identified by differential display often do not give a  
signal on Northern blots even after several days.  
Differential expression was, in this case, confirmed by  
30 Rnase protection and semi-quantitative PCR with sequence  
specific primers. These observations are consistent with  
the theoretical expectation that, because of the difficulty  
20 of driving hybridization of low abundance cDNA to  
completion, such sequences will be more readily identified  
35 by PCR based differential display than by hybridization  
based RDA. There may, in addition, be another reason for  
the greater sensitivity of modified differential display.  
40 It has been reported (Meyuhas and Perry, 1979, Cell 16:  
25 139-148) that mRNA species of low abundance are on average  
twice the size of smaller, more stable and more abundant  
mRNA species. It is, therefore, more likely that both  
45 members of a pair of arbitrary primers will hybridize to  
and detect differentially expressed cDNA from the longer  
(average 4.9 kb) very diverse 80% of mRNA species that are  
30 represented by very few copies per cell than from the



5 shorter (average 2 kb) 20% of mRNA species that are more abundantly expressed.

10 In preliminary experiments, an average of three differentially displayed bands were identified for each pair of primers. With a total of 66 primer pairs generated  
5 from all possible combinations of 12 independent primers, approximately 200 gene fragments could be identified. In some cases multiple fragments may derive from the same  
15 gene. Fig. 7 shows the pattern of differential display fragments observed with one pair of arbitrary decamers, MR\_1 (TAC AAC GAG G) and MR\_5 (GGA CCA AGT C). A number of  
20 bands can be identified that are associated with all four tumors but not with the parental cells. This distribution is unrelated to the immunogenicity of the tumor cells, since only three of the four tumors are immunologically crossreactive. In contrast to the differentially expressed  
25 bands identified by RDA, which gave positive results on Northern blots exposed for only a few hours, fragments identified by differential display did not give a signal on Northern blots even after several days. Differential  
30 expression of the differential display fragments can, however, be confirmed by RNase protection assays or by semi-quantitative PCR with sequence specific primers. An  
35 example is shown in Fig. 8, the results of an RNase protection assay with clone 90 from differential display band 9. This sequence, which has no significant homology to entries in the GenBank database, is expressed in all four tumor lines but not in the parental B/c.N.

40 As discussed above, we attribute this striking  
25 difference in the results of RDA and differential display to the greater sensitivity of the PCR based modified differential display as compared to the hybridization based  
45 RDA method. Based on the pattern of expression in the different tumor and normal cell lines, it appears that the shared tumor antigen detected following direct immunization  
30 of mice with syngeneic tumor cells may be encoded by a more abundantly expressed IAP gene. The methods described in  
50

5 this example can be used to determine whether the products  
of the less abundantly expressed genes identified by  
modified differential display represent potential cryptic  
10 tumor antigens.

5 9.5. SELECTION OF FULL LENGTH cDNA  
ENCODING POTENTIAL TUMOR IMMUNOGENS

15 This section presents methods for facilitating  
selection of corresponding full length cDNAs from fragments  
of differentially expressed genes identified by  
representational difference analysis or by modified  
10 differential display (Fig. 9). A single stranded  
biotinylated probe is synthesized from isolated cDNA  
fragments and is used to select the longer cDNA that  
contain a complementary sequence by solution hybridization  
20 to single stranded circles rescued from a phagemid tumor  
cDNA library. This method is especially well-suited to the  
15 use of DNA fragments isolated by the modified differential  
display method employing two arbitrary primers. The same  
arbitrary primers employed for PCR amplification of a given  
30 fragment in differential display can be modified to  
generate a single stranded hybridization probe from that  
20 fragment. This avoids the need to sequence, select and  
synthesize a new pair of fragment specific primers for each  
35 new fragment of interest.

i) The two oligonucleotides of a pair of PCR primers  
employed in differential display are modified:  
40 (biotin-dT)-dT- (biotin-dT) is incorporated at the 5' end  
25 of one primer and a phosphate is incorporated at the 5' end  
of the second primer. These modified primers are  
incorporated by PCR into the two strands of a differential  
display fragment that was selected following the original  
45 PCR amplification with the same unmodified arbitrary  
primers. From this double stranded PCR product, the strand  
labelled with a 5' phosphate is digested with  $\lambda$  exonuclease  
30 to generate a single stranded biotin-labeled probe.

5           ii) Single stranded (ss) DNA circles are rescued from  
a phagemid cDNA library using the M13K07 packaging  
defective phage as helper virus. This library is  
10       constructed in the pcDNA3.1/Zeo(+) phagemid (Invitrogen,  
Carlsbad, CA) with insertion of (ApaI)oligo-dT primed cDNA  
5       between the Apa I and Eco RV restriction sites. A key  
manipulation to achieve the efficient ligation necessary  
for construction of a high titer cDNA library is to insure  
15       that cDNA inserts are 5' phosphorylated by treating with T4  
polynucleotide kinase prior to ligation. The  
biotin-labeled single stranded probe generated from the  
10       differential display fragment is hybridized in solution to  
the ssDNA circles of the phagemid library. The  
20       biotin-labeled hybridization complexes can then be  
separated from unrelated ssDNA on streptavidin magnetic  
beads and the ss circles eluted for further analysis (Fig.  
25       9).

15           As a test of this enrichment method, a model  
plasmid mix was prepared that included 1% of a specific  
arbitrarily selected recombinant clone, 3f IAP. A  
30       biotinylated ss-probe was prepared from the 3f RDA fragment  
and used to select single stranded phagemid circles from  
the 1% plasmid mix. Following elution from streptavidin  
20       beads, the single stranded circles were hybridized to a  
sequence specific oligonucleotide in order to prime  
35       synthesis of the second plasmid strand prior to bacterial  
transformation. Plasmid DNA was prepared from 63  
transformed colonies. 63 of 63 of these plasmid  
40       preparations expressed the target 3F IAP insert. This  
25       method therefore appears to be very efficient.

The same method appears to work with similar  
efficiency in the more stringent case of a differential  
45       display fragment (B4) representing a previously  
unidentified sequence that is expressed in all four murine  
tumors at a concentration approximately 10 fold greater  
30       than in the non-tumorigenic parental cells. 5 out of 5  
50       transformants randomly picked following selection of single

strand circles with the 200 bp B4 DNA fragment had longer inserts that were positive by PCR with sequence specific primers. This method therefore appears to be very efficient.

10. **EXAMPLE: INDEPENDENT HUMAN TUMOR CELL  
LINES DERIVED FROM A NON-TUMORIGENIC,  
IMMORTALIZED CELL LINE**

The following example describes a set of human tumors independently derived by different carcinogens or oncogene transformation from the same cloned, non-tumorigenic parental cell line. As in the previous examples of the use of RDA and modified differential display for identification of gene products differentially expressed in murine tumors, the availability of related normal and tumor cell lines has considerable advantages for the molecular and immunological analysis of potential cancer vaccines. This not only provides a readily available source of normal control cells and RNA, but also makes it possible to focus on molecular features that are carcinogen independent and, since they are shared by multiple independent tumors, are unlikely to be the products of random genetic drift during *in vitro* propagation.

A set of human uroepithelial tumors have been derived in the laboratory of Dr. Catherine Reznikoff (University of Wisconsin, Madison) from an SV40 immortalized human uroepithelial cell line, SV-HUC, that is itself contact inhibited, anchorage dependent and non-tumorigenic in nude mice (Christian, et al., 1987, Cancer Res. 47: 6066-6073). A series of independent tumor cell lines were derived by either *ras* transformation (Pratt, et al., 1992, Cancer Res. 52: 688-695) or *in vitro* mutagenesis of SV-HUC with different carcinogens including some that are bladder-specific (Bookland, et al., 1992, Cancer Res. 52: 1606-1614). Transformed cells were

initially selected on the basis of altered *in vitro* growth requirements and each was shown to be tumorigenic in nude mice. A subset of these tumors is selected that retain the phenotype of transitional cell carcinoma. Table 7 lists the parental cells and the carcinogens employed to derive these 5 tumor lines *in vitro*. A systematic program is undertaken to 1) identify full length cDNA differentially expressed in these tumors and 2) to test the immunogenicity in HLA and human CD8 transgenic mice of these cDNA products cloned into a vaccinia virus expression vector.

10

---

TABLE 7: HUMAN UROEPITHELIAL CELL LINES

Acquired from Dr. Catherine A. Reznikoff, University of Wisconsin Clinical Cancer Center

Parental Line	Immortalization
SV-HUC	SV40 immortalized normal bladder epithelial cells
Tumor Line	Carcinogen or Oncogene transformation
MC pT7	3-methylcholanthrene
MC ppT11-A3	3-methylcholanthrene followed by 4-aminobiphenyl
MC ppT11-HA2	3-methylcholanthrene followed by N-hydroxy-4-acetylaminobiphenyl
HA-T2	N-hydroxy-4-aminobiphenyl
SV-HUC/ <i>ras</i> -T	EJ/ <i>ras</i>

---

Experiments apply both representational difference analysis and modified differential display to identify gene fragments differentially expressed in the MC ppT11-A3 tumor (ppT11A3) relative to the parental SV-HUC. All differentially expressed fragments are tested by Northern analysis and RNase protection assay for parallel

5 expression in mRNA of the other tumor cell lines. Only  
those DNA clones expressed in at least 3 of the 5 SV-HUC  
derived tumor cell lines are selected for further  
10 characterization.

Similar analysis of tumor-specific gene products  
5 can be carried out with tumors derived from SV40 large T or  
HPV E6 or E7 immortalized cell lines representative of  
other human tissues. Published examples include: prostatic  
15 epithelium (Parda et al., 1993, The Prostate 23: 91-98, ),  
mammary epithelium (Band et al., 1990, Cancer Res. 50:  
7351-73-57), and bronchial epithelium (Gerwin et al., 1992,  
10 Proc. Natl. Acad. Sci. USA 89: 2759-2763; Klein-Szanto et  
al., 1992, Proc. Natl. Acad. Sci. USA 89: 6693-6697).

11. **EXAMPLE: GENE EXPRESSION IN FRESH**  
**PATIENT BLADDER TUMORS**

25 The above-described methods for identification of  
15 differentially expressed genes require that both tumor and  
normal control cell mRNA be readily available. The  
preceding section focuses on tumors derived in vitro from  
30 immortalized cell lines, from which mRNA may be readily  
obtained in large quantities.

20 In spite of the advantages of working with in  
vitro-derived tumors from which mRNA may be readily  
35 obtained, it is necessary to address the possibility that  
some transformation-associated gene expression might be  
missed or, conversely, that some differential gene  
40 expression detected might not be transformation related.  
25 Although the normal control is contact inhibited, anchorage  
dependent and non-tumorigenic, it is likely that it has  
undergone some pre-neoplastic event that is the basis for  
continuous growth in vitro. Perhaps a greater concern is  
45 that extraneous gene expression associated with in vitro  
proliferation might be identified. Two strategies to  
30 exclude such events are employed. First, genes are

5 analyzed that are expressed in at least 3 of the 5 bladder  
tumor lines but that are not expressed in the *in vitro*  
10 adapted parental cells. This will a) filter out any  
systematic gene expression selected by *in vitro* growth,  
5 since this should be shared by the normal parental cells;  
and b) identify any alterations in gene expression that  
are carcinogen specific or that may arise as a result of  
15 random genetic drift during *in vitro* propagation, since it  
is not expected that these would be shared by multiple  
independent tumors derived by diverse carcinogens (or  
20 oncogene transformation). Second, and most important, only  
those differentially expressed genes that can also be shown  
to be expressed in multiple samples of fresh patient tumor  
material are selected for further characterization.

Patient tumor material together with normal  
25 bladder epithelium is cryopreserved following surgery. In  
comparison to some other carcinomas, normal tissue control  
15 is readily available from bladder cancer patients. Total  
RNA is extracted from frozen samples by the acid  
guanidinium isothiocyanate method (Lee and Costlow, 1987,  
30 Methods in Enzymology 152:633-648). Following Dnase I  
treatment, polyA mRNA is fractionated on oligo dT beads and  
20 gene expression is analyzed by Northern blot, RNase  
protection assay, and semi-quantitative RT/PCR. For each  
35 differentially expressed gene fragment identified in the *in*  
*vitro* tumor lines, expression of the gene is characterized  
in a panel of 20 patient tumors and normal tissue controls.  
40 This sample size permits the estimation of the proportion  
25 of patients expressing the gene with a standard error no  
greater than 0.11% ( $SE = \sqrt{p \cdot (1-p)/n}$  where  $p = \text{true}$   
proportion and  $n = \text{sample size}$ . SE is maximal for  $p = 0.5$ , at  
45 that proportion, 10/20 patients,  $SE = \pm 0.11$ ; for any other  
value of  $p$ , SE is smaller.) Expression of some of these  
genes may be correlated in the different tumor samples.  
30 This is useful because it creates the possibility of

multiple T cell epitopes that could associate with different human MHC molecules.

The expression pattern is also determined, in other normal adult and fetal tissues, of any gene that is differentially expressed in bladder tumors relative to normal bladder epithelium. Total RNA or first strand cDNA prepared from over 30 different human normal adult or fetal tissues (Discovery Line™ RNA and Gene Pool™ cDNA, Invitrogen, Carlsbad, CA) is used. Expression in fetal but not normal adult tissue is particularly interesting and does not preclude consideration as an immunotherapeutic reagent. Expression of intermediate abundance species are determined by Northern analysis. Low abundance species are quantitated by RNase protection assay and semi-quantitative PCR. Those sequences that are recurrently expressed in tumors derived from multiple patients and which have the lowest relative expression in normal tissue are selected for further characterization as potential tumor-specific antigens.

12. EXAMPLE: THE USE OF DIFFERENTIALLY  
EXPRESSED GENE PRODUCTS TO GENERATE

CTLs

CROSSREACTIVE WITH AUTHENTIC TUMORS

To identify differentially expressed gene products that might be candidates for tumor immunotherapy, it is necessary to have a means of delivering the product for immunization in an environment in which T cell responses to peptides associated with human HLA can be induced. T cells induced by immunogenic products could then be tested for crossreactivity on HLA compatible tumors that express the corresponding mRNA. This example describes the use of HLA and human CD8 transgenic mice for induction of T cell responses to peptides associated with human HLA. If all these conditions are met: 1) the gene is differentially expressed in multiple human tumors but



5 not normal tissue counterparts; 2) gene products are  
immunogenic in association with HLA; and 3) the specific T  
10 cells induced are crossreactive on human tumor cells, then  
this would constitute key preliminary data preparative to  
initiation of clinical vaccine trials.

5 To determine whether the products of  
differentially expressed genes are immunogenic, groups of  
three (HLA-A2.1 x huCD8)F<sub>1</sub> transgenic mice are immunized  
15 intravenously with 5 x 10<sup>6</sup> pfu of each specific recombinant  
vaccinia virus (Bennink and Yewdell, 1990, Current Topics  
in Microbiol. and Immunol. 163: 153-178). After at least  
20 two weeks, mice are sacrificed and CD8<sup>+</sup> splenic T cells are  
enriched on anti-CD8 coated magnetic beads. CD8<sup>+</sup> cytolytic  
precursors are restimulated in vitro with parental SV-HUC  
cells that are transfected with the recombinant  
25 differentially expressed gene previously isolated in the  
pcDNA3.1/Zeo(+) plasmid expression vector (Section 9.3).  
15 Substitution of the plasmid recombinant in place of the  
vaccinia vector for restimulation in vitro is necessary to  
avoid a large vaccinia vector specific response. After  
30 five days in vitro culture, cytolytic activity is  
determined by <sup>51</sup>Cr release from SV-HUC target cells  
20 transfected with either the specific recombinant plasmid or  
a control ovalbumin gene recombinant.

35 This same cytolytic assay can be readily applied  
to determine whether the relevant CTL epitope is also  
presented by HLA compatible tumor cells that express the  
40 corresponding mRNA. If T cells are induced in (HLA-A2.1 x  
25 huCD8)F<sub>1</sub> transgenic mice, HLA compatible targets include  
tumor cells that either express native HLA-A2.1 or that  
have been transfected with HLA-A2.1. The immunogenicity of  
45 differentially expressed gene products is established and  
it is determined whether there is a crossreaction with  
human tumor cells. This finding, together with the  
30 demonstration that the same mRNA is expressed in multiple  
samples of fresh patient tumors but not normal tissues  
50

5 (Section 11), is required prior to initiation of a clinical vaccine trial.

10 An important consideration for vaccine development is the extensive polymorphism of human class I HLA. As discussed above, an appealing strategy is to  
5 target four major HLA subtypes, A2, A3, B7 and B44, that provide broad coverage across ethnic populations. Many peptides bind to multiple members of a single subtype. If  
15 several CTL epitopes are identified for each subtype, then this can greatly facilitate formulation of a broadly effective vaccine.

10  
20 **13. EXAMPLE: INDUCTION OF PROTECTIVE IMMUNITY**

It is desirable, especially in the case of cryptic tumor antigens encoded by low abundance mRNA, to determine whether a T cell response to differentially  
25 expressed gene products confers protective tumor immunity. Since a number of differentially expressed genes have been identified in the murine tumor model described above, such experiments are carried out in mice.

30 It has previously been reported for this murine tumor model (Sahasrabudhe, et al., 1993, J. Immunology 151: 6302-6310) that three of four independently derived tumors  
20 are immunologically crossreactive. Many of the differentially displayed bands identified in these tumors are, in contrast, present in all four tumors. It is, therefore, unlikely that the genes from which these fragments derive are immunologically dominant in animals  
35 inoculated with these tumors.

40  
25 If it is shown that direct immunization with a recombinant differentially expressed gene does, nevertheless, confer protective immunity, then this provides compelling evidence for the efficacy of  
45 vaccination with a cryptic tumor antigen.

30 Groups of 5 mice of the BALB/c strain syngeneic to the murine tumors are immunized with each vaccinia virus

5 recombinant for a full length cDNA differentially expressed  
in all four murine tumor lines but not the parental B/c.N  
cells (Fig. 7). Each group of mice is assayed for  
10 induction of protective immunity by challenge with a  
tumorigenic inoculum of  $1 \times 10^6$  BCA 39 tumor cells  
5 (Sahasrabudhe, et al., 1993, J. Immunology 151:6302-6310).  
To determine whether protective immunity correlates with  
relative quantitative expression, independent gene products  
15 are tested that represent different levels of differential  
expression as determined by semi-quantitative PCR.

10 14. EXAMPLE: CONSTRUCTION AND  
20 CHARACTERIZATION OF VACCINIA  
EXPRESSION VECTORS FOR USE IN VACCINES

This example describes the construction and  
characterization of a new set of direct ligation vectors  
25 designed to be universally applicable for the generation of  
15 chimeric vaccinia genomes. The aim was to modify the  
genome of vNotI/tk so as to acquire direct ligation vectors  
which are more universally useful. First, the insertion  
30 site was changed by placing the sites for two unique  
restriction enzymes at the beginning of the thymidine  
kinase gene. This allows one to fix the orientation of the  
20 insert DNA and eliminates the production of contaminating  
35 wild type genomes after religation of viral arms. Second,  
in order to generate a direct ligation vector which would  
express high levels of protein, the thymidine kinase gene  
was preceded by a strong constitutive vaccinia virus  
40 promoter.

25 These new ligation vectors contain a pair of  
unique restriction sites, NotI and ApaI, to eliminate  
religation of poxvirus arms and fix the orientation of the  
45 insert DNA behind strongly expressing constitutive vaccinia  
promoters. The insertion cassette has been placed at the  
beginning of the thymidine kinase gene in vaccinia to  
30 utilize drug selection in the isolation of recombinants.

14.1. MATERIALS AND METHODS14.1.1. PLASMID CONSTRUCTION

Pairs of oligonucleotides were constructed which, when annealed, contained the 7.5k gene promoter

(MM436:GGCCAAAATTGAAAACTAGATCTATTTATTGCACGCGGCCCATGGGCC C (SEQ ID NO.:φφ) and MM437:

GGCCGGGCCCATGGCGCGCGTGCAATAAATAGATCTAGTTTTTCAATTTTT (SEQ ID NO.: φφ)), or the synthetic EL promoter

(MM438:GGCCAAAATTGAAATTTATTTTTTTTTTTTGGGAATATAAAGCGGCCGCCA T GGGCCC (SEQ ID NO.:φφ) and MM439:

GGCCGGGCCCATGGCGCGCGCTTATATTCCAAAAAATAAAATTTCAATTTTT (SEQ ID NO.:φφ)) and restriction sites for NotI and ApaI.

The double-stranded oligonucleotides were annealed by ramping from 94° C to 20° C over two hours and ligated into the NotI site present in pJNotI/tk, a plasmid containing the HindIII J fragment from vNotI/tk, resulting in plasmids p7.5/tk and pEL/tk.

A Polymerase Chain Reaction (PCR) was performed on pBI221, a plasmid containing the *E.coli gusA* gene encoding for β-glucuronidase (β-glu), using primers MM440 (GGGAAAGGGGCGGCCCATGTTACGTCCTGTAGAAACC) (SEQ ID NO.:φφ) and MM441 (GGGAAAGGGGCGGCCCTCATTGTTGCCTCCCTGCTG) (SEQ ID NO.:φφ), or MM440 and MM442

(GGGAAAGGGGCGGCCCTCATTGTTGCCTCCCTGCTG) (SEQ ID NO.:φφ), and the resulting fragment was cloned into pCRII (TA cloning kit, Invitrogen). The plasmids were excised with NotI (MM440/MM442 product) and cloned into pJNot/tk digested with NotI yielding pJNot/tk-GUS, or excised with NotI and ApaI (MM440/MM441 product), and inserted into pEL/tk and p7.5/tk previously digested with ApaI and NotI yielding p7.5/tk-GUS and pEL/tk-GUS.

Pairs of oligonucleotides were constructed which, when annealed, contained the 7.5k gene promoter and the nucleotide sequence encoding for a cytotoxic T-cell epitope for ovalbumin (11) (SIINFELK; SEQ ID NO:φφ) (75ova:

GGCCAAAATTGAAAACTAGATCTATTTATTGCACCATGAGTATAATCAACTTTGAAA

5 A ACTGTAGTGA (SEQ ID NO.: $\phi\phi$ ) and 75ovarv:  
 GGCCTCACTACAGTTTTTCAAAGTTGATTAATACTCATGGTGCAATAAATAGATCTAGT  
 10 T TTTCAATTTTT (SEQ ID NO.: $\phi\phi$ ) or the EL promoter and the  
 peptide SIINFELK (SEQ ID NO:  $\phi\phi$ ) (ELova:  
 GGCCAAAATTGAAATTTTATTTTTTTTTTTTGGGAATATAAACCATGAGTATAATCAAC  
 5 T TTGAAAACCTGTAGTGA (SEQ ID NO.: $\phi\phi$ ) and ELovarv:  
 GGCCTCACTACAGTTTTTCAAAGTTGATTATACTCATGGTTTATATTCCAAAAA  
 15 A ATAAATTTCAATTTTT (SEQ ID NO.: $\phi\phi$ ). The double-stranded  
 oligonucleotides were annealed by ramping from 94° C to 20°  
 C over two hours and ligated into the NotI site present in  
 pJNotI/tk, a plasmid containing the HindIII J fragment from  
 10 vNotI/tk resulting in plasmids p7.5/tk-ova and pEL/tk-ova.

#### 14.1.2. GENERATION OF RECOMBINANT VIRUSES

Cells and viruses were maintained and manipulated  
 as described by Earl, et al. (1991, In Ausubel, et al.,  
 25 (eds.), Current Protocols in Molecular Biology. Greene  
 Publishing Associates/Wiley Interscience, New York).  
 Recombinant viruses were made using homologous  
 30 recombination by infecting CV-1 cells at a multiplicity of  
 infection (moi) of 0.05 and two hours later transfecting  
 DNA into the infected cells using lipofectamine (Life  
 Technologies Incorporated) as suggested by the  
 20 manufacturer. After 72 hours the cells were harvested and  
 isolated plaques were selected by passage in Hutk cells in  
 the presence of bromodeoxyuridine (Earl, et al., 1991, In  
 Ausubel, et al. (eds.), Current Protocols in Molecular  
 40 Biology. Greene Publishing Associates/Wiley Interscience,  
 25 New York) or HAT supplemented media (Weir, et al., 1982,  
 Proc. Nat. Acad. Sci. USA, 79:1210-1214).

Vaccinia virus was generated from viral DNA by  
 rescue with fowlpox virus (Scheifflinger, et al., 1992,  
 45 Proc. Natl. Acad. Sci. USA 89:9977-9981). Vaccinia virus  
 was isolated from infected HeLa cells by banding and  
 30 sedimentation in sucrose (Earl, et al., 1991, In Ausubel,  
 et al. (eds.), Current Protocols in Molecular Biology.

5 Greene Publishing Associates/Wiley Interscience, New York).  
The purified virions were treated with Proteinase K  
(Boehringer Mannheim) and gently extracted with buffer  
10 saturated phenol, phenol:chloroform (50:50), and chloroform  
before precipitation with 2.5 volumes of ethanol in 0.3M  
5 sodium acetate and resuspended in TE (10mM TrisHCl, pH8.0.  
1mM EDTA (Earl, et al., 1991, In Ausubel, et al. (eds.),  
15 Current Protocols in Molecular Biology. Greene Publishing  
Associates/Wiley Interscience, New York). Confluent wells  
of BSC-1 cells from a 12 well dish were infected with  
fowlpox virus and after a two hour incubation at 37° C were  
10 transfected with 0.6 µg full length vaccinia DNA using  
Lipofectamine (Life Technologies Incorporated) as suggested  
by the manufacturer. After 24, 48, and 72 hours the cells  
were harvested, lysed by three freeze-thaw cycles and  
20 screened by plaque assay on BSC-1 cells (Earl, et al.,  
1991, In Ausubel, et al., (eds.), Current Protocols in  
15 Molecular Biology. Greene Publishing Associates/Wiley  
Interscience, New York).

30 14.1.3. GENERATION OF RECOMBINANT  
VIRUSES BY DIRECT LIGATION

20 The 1.1 kB Eco RI/ Eco RV restriction  
35 endonuclease fragment containing ovalbumin from pHbeta -  
Ova-neo (Pulaski, et al., 1996, Proc. Natl. Acad. Sci. USA,  
93:3669-3674) was inserted into the EcoRI and EcoRV sites  
of pBluescript KS+ (Stratagene), generating pBS.ova. The  
40 DNA product from a Polymerase Chain Reaction (PCR) on  
25 pBS.ova using primers VV0LZ5  
(GCAGGTGCGCCGCGCGTGGATCCCCCGGCTGCAGG) (SEQ ID NO.: φφ) and  
VVTLZ3 (GTACCGGGCCCAAAAACAAAATTAGTTAGTTAGGCCCCCCTCGA)  
45 (SEQ ID NO.: φφ) was digested with ApaI and NotI (Life  
Technologies, Inc.), gel purified from low melting point  
agarose (Bio-Rad) using beta Agarase (Life Technologies,  
30 Inc.) following the recommendations of the manufacturer,  
50 and cloned into pBluescript KS+ that had been digested with

5 NotI and ApaI, generating pBS.VVova. A DNA fragment  
encoding ovalbumin was excised from pBS.VVova by digestion  
of this plasmid with ApaI and NotI and purified after  
10 electrophoresis through a low melting point agarose gel  
using beta Agarase. One microgram of purified vEL/tk DNA  
5 was digested with ApaI and NotI and centrifuged through a  
Centricon 100 concentrator (Amicon) to remove the small  
intervening fragment. The vEL/tk DNA arms and the DNA  
15 fragment encoding ovalbumin were ligated overnight at room  
temperature, at a 4:1 (insert: virus) molar ratio, in 30  
microliters with 5 units T4 DNA Ligase. The ligation  
20 product was transfected using lipofectamine (Life  
Technologies, Inc.) into a well of confluent BSC-1 cells  
from a 12 well plate two hours after infection with fowlpox  
virus at 1 pfu/cell. Three days later the cells were  
25 harvested and isolated plaques were selected by passage in  
Hutk- cells in the presence of bromodeoxyuridine (Earl, et  
15 al., 1991 In Ausubel, et al. (eds.), Current Protocols in  
Molecular Biology. Greene Publishing Associates/Wiley  
Interscience, New York).

#### 14.1.4. ANALYSIS OF VIRAL DNA GENOMES

20 BSC-1 cells were infected at high multiplicity of  
infection (moi) by vaccinia WR, vEL/tk, v7.5/tk, or  
35 vNotI/tk. After 24 hours the cells were harvested and  
resuspended in Cell Suspension Buffer (Bio-Rad Genomic DNA  
Plug Kit) at  $1 \times 10^7$  cells/ml. An equal volume of 2% CleanCut  
40 agarose (Bio-Rad) preincubated at 50° C was added and the  
cell suspension was formed into 100  $\mu$ l plugs. After  
25 hardening at 4° C the plugs were treated as previously  
described to digest protein (Merchlinsky, et al., 1989. J.  
Virol. 63:1595-1603). The plugs were equilibrated in the  
45 appropriate restriction enzyme buffer and 1mM PMSF for 16  
hours at room temperature, incubated with restriction  
enzyme buffer, 100ng/ml Bovine Serum Albumin and 50 units  
30

5 NotI or ApaI for two hours at 37° C (NotI) or room temperature (ApaI) prior to electrophoresis.

10 One well of a 6 well dish of BSC-1 was infected with v7.5/tk or vEL/tk at high multiplicity of infection (moi) and after 48 hours the cells were harvested, pelleted  
5 by low speed centrifugation, rinsed with Phosphate-Buffered Saline (PBS), and the DNA was isolated using DNazol (Gibco). The final DNA product was resuspended in 50  
15 microliters of TE (10mM TrisHCl, pH8.0. 1mM EDTA) and 2.5 microliters were digested with HindIII, HindIII and ApaI, or HindIII and NotI, electrophoresed through a 1.0% agarose  
20 gel, and transferred to Nytran (Schleicher and Schuell) using a Turboblotter (Schleicher and Schuell). The samples were probed with p7.5/tk (Figure 11a) or pEL/tk (Figure 11b) labeled with <sup>32</sup>P using Random Primer DNA Labeling Kit (Bio-Rad) in QuickHyb (Stratagene) and visualized on Kodak  
25 XAR film.

15 One well of a 6 well dish of BSC-1 cells was infected with v7.5/tk, vEL/tk, vNotI/tk, vpNotI, vNotI/lacZ/tk, or wild type vaccinia WR at high  
30 multiplicity of infection (moi) and after 48 hours the cells were harvested, pelleted by low speed centrifugation, rinsed with Phosphate-Buffered Saline (PBS), and the DNA  
20 was isolated using DNazol (Gibco). The final DNA product was resuspended in 50 microliters of TE (10mM TrisHCl, pH8.0. 1mM EDTA) and used in a PCR (30 cycles, 1 minute 94°  
35 C, 2 minutes 55° C, 3 minutes 72° C, MJ Research PTC-100) with primers MM407 (GGTCCCTATTGTTACAGATGGAAGGGT) (SEQ ID NO.:  $\phi\phi$ ) and MM408 (CCTTCGTTTGCCATACGCTCACAG) (SEQ ID NO.:  
40  $\phi\phi$ ). The nucleotide sequence was determined by <sup>35</sup>S sequencing using Sequenase Version 2.0 DNA Sequencing Kit (Amersham), and visualized after electrophoresis through 8%  
45 denaturing polyacrylamide gels by exposure to Bio-Max film (Kodak).

30



#### 14.1.5. DETERMINATION OF $\beta$ -GLUCURONIDASE ACTIVITY

A well of BSC-1 cells from a 12 well plate was infected at an moi of 1 with vNotI/tk-GUS, v7.5/tk-GUS and vEL/tk-GUS, the cells were harvested 20 hours post infection, resuspended in 0.5ml PBS, and disrupted by three cycles of freeze-thawing. The extract was clarified by a short microfuge spin (one minute, 14,000 rpm) and the supernatant was analyzed for  $\beta$ -glu units as described by Miller, 1972, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY as adapted for 96-well plates. The  $A_{405}$  values were determined on a microplate reader (Dynatech MR3000) and the  $\beta$ -glu activity was determined by comparison to  $\beta$ -glu (Clontech) standards analyzed in the same assay.

#### 14.1.6. ANALYSIS OF CYTOXIC T CELL RESPONSE

Confluent monolayers of MC57G cells in wells of a 6 well plate were infected at an moi of 1 with vEL/tk, v7.5/tk-ova, vEL/tk-ova, vEL/tk-ovaFL clone 1, and vEL/tk-ovaFL clone 2 (vEL/tk-ovaFL are virus clones of full length ovalbumin generated by direct ligation). At 16 hours post infection cells were harvested, labeled with 100 microcuries  $^{51}\text{Chromium}$  (Dupont) for 1 hour at 37° C, and 10<sup>4</sup> cells were added to wells of a 96 well round bottom plate in quadruplicate. A sample of uninfected MC57G cells incubated with 1 micromolar purified ova 257-264 peptide was also incubated with  $^{51}\text{Cr}$  as a positive control and untreated MC57G cells were used as a negative control. T cells specific for ova 257-264 were added to target cells at ratios of 2:1 and 10:1. Cells were incubated at 37° C for 4 hours, supernatants were harvested, and  $^{51}\text{Cr}$  release determined. Spontaneous release was derived by incubating target cells with media alone and maximal release was determined by incubating target cells with 5% Triton X 100. Percentage of specific lysis was calculated using the formula: % specific lysis = ((experimental release -

spontaneous release) / (maximal release-spontaneous release)) X 100. In each case the mean of quadruplicate wells was used in the above formula.

## 14.2. RESULTS

### 14.2.1. CONSTRUCTION OF DIRECT LIGATION VECTORS

The vaccinia WR genome is approximately 190 kilobases in length and rich in A and T residues. The complete sequence of the vaccinia WR genome was provided by P. Earl of the Bernard Moss laboratory (Laboratory of Viral Diseases, NIAID, NIH, Bethesda, MD). A restriction enzyme search of the complete sequence of the vaccinia WR genome using MacVector (IBI) revealed a lack of restriction sites for ApaI, AscI, BspI20I, FseI, RsrII, SfiI, SrfI and SgfI. The ready availability of highly active and pure preparations of the enzyme as well as the generation of a staggered end upon digestion led us to choose to use ApaI as the second site in conjunction with the NotI site already present in vNotI/tk.

Vaccinia virus based expression vectors are most useful when the foreign protein is expressed constitutively. The expression of foreign proteins during the early stage of viral replication is essential for cytotoxic T cell response (Bennick, et al. 1990, Topics Microbiol. Immunol. 163:153-184) and high levels of total protein expression have been observed using promoters active during the late stage of viral replication. We decided to incorporate the promoters corresponding to the constitutively expressed 7.5k gene (Mackett, et al., 1984, J. Virology, 49:857-864) and a constitutively expressed synthetic promoter EL noted for high level expression.

A useful feature of vNotI/tk that must be retained in any new vector is the ability to discriminate for recombinant viral genomes using selection against an active thymidine kinase gene. The introduction of the ApaI site within the coding sequence for the tk gene

5 necessitates an increase in the total number of amino acids  
in order to accommodate the restriction enzyme site. A  
10 comparison of the amino acid sequence for thymidine kinase  
genes from a variety of animal and viral species showed the  
region of greatest heterogeneity was at the N terminus of  
5 the protein, suggesting that this region of the protein  
could tolerate a modest increase in the number of amino  
15 acids.

The recombination-independent cloning vectors  
were constructed by making plasmid intermediates containing  
the modified thymidine kinase (tk) gene and replacing the  
10 tk sequence in the vNotI/tk genome by homologous  
recombination. Two sets of oligonucleotide pairs were  
20 constructed which, when annealed, contained the promoter  
for the 7.5k gene or the synthetic EL sequence and  
restriction sites for NotI and ApaI. The modified  
25 thymidine kinase genes were constructed by annealing the  
15 double-stranded oligonucleotides and ligating the product  
into the NotI site present at the beginning of the  
thymidine kinase gene in pJNotI/tk, a plasmid containing  
30 the HindIII J fragment from vNotI/tk. The oligonucleotide  
pairs annealed to and eliminated the NotI site in pJNotI/tk  
generating a new NotI site closely followed by an ApaI site  
20 after the promoter and flanking the nucleotides coding for  
35 the initial methionine in the thymidine kinase gene  
resulting in plasmids p7.5/tk and pEL/tk (Figure 1). The  
acquisition of the ApaI site was verified by restriction  
enzyme analysis of plasmid DNA and the nucleotide sequence  
40 of the thymidine kinase gene promoter was determined and  
25 found to be as depicted in Figure 1.

The recombinant viruses derived from p7.5/tk and  
pEL/tk were isolated using a strategy relying on positive  
45 drug selection in the presence of HAT (hypoxanthine,  
aminopterin, thymidine) (Weir, et al., 1982, Proc. Nat.  
Acad. Sci. USA 79:1210-1214). The viruses vpNotI, a virus  
30 that contains a copy of pBR322 inserted at the NotI site of  
vNotI/tk (Merchinsky, et al., 1992, Virology 190:522-526),  
50

5 and vNotI/lacZ/tk, a virus with a copy of the lacZ gene  
interrupting the thymidine kinase in vNotI<sup>-</sup> (Merchlsinsky, et  
al., 1992, Virology. 190:522-526) are thymidine kinase  
10 negative (tk<sup>-</sup>) viruses that are identical to vNotI/tk except  
for the inserted DNA at the beginning of the tk gene. The  
5 plasmids p7.5/tk and pEL/tk were recombined with vpNotI and  
vNotI/lacZ/tk helper viruses in CV-1 cells and the infected  
monolayers were harvested and passaged in the presence of  
15 HAT media on Hutk<sup>-</sup> cells. Individual plaques were passaged  
and isolated an additional three rounds on Hutk<sup>-</sup> cells  
before expansion and analysis.

#### 10 14.2.2. ANALYSIS OF THE STRUCTURE OF THE VIRAL GENOMES

The growth of v7.5/tk and vEL/tk virus in HAT  
supplemented media implies these viruses, in contrast to  
vpNot and vNot/lacZ/tk, contain an active thymidine kinase  
25 (tk) gene. However, an active tk gene could arise from  
multiple crossovers which delete the 7.5k or EL promoter  
sequences, generating a virus with the normal tk promoter.  
The v7.5/tk and vEL/tk genomes should contain a unique site  
30 for both NotI and ApaI within the HindIII J fragment. The  
genomic structure of the isolated virus stocks was analyzed  
by restriction enzyme digestion of DNA in agarose plugs  
20 derived from virus infected cells using NotI or ApaI and  
electrophoresis of the products through 1% agarose (Figure  
35 10). Uncut vaccinia WR (lane 2) migrates at a size of 190  
kilobase pairs as compared to multimers of bacteriophage  
lambda (lane 1). After digestion with NotI vaccinia WR is  
40 cleaved into two fragments approximately 150 and 40  
kilobase pairs in length (7th lane from left) whereas the  
25 vNot/tk, vEL/tk, and v7.5/tk were cleaved into fragments of  
about 110 and 80 kilobase pairs. When the same samples  
45 were digested with ApaI, only one fragment the size of the  
uncut genome was observed for both vaccinia WR and vNot/tk  
while vEL/tk and v7.5/tk gave the same sized fragments  
30 observed after digestion with NotI. Therefore, both

5 v7.5/tk and vEL/tk contain a unique site for both ApaI and  
NotI, the sites are at the same locus as the NotI site in  
10 vNot/tk, and the sites are in a more central location in  
the genome than the HindIII F fragment which contains the  
NotI site in vaccinia WR. The background of cellular DNA  
5 fragments was more pronounced in the ApaI digestion, which  
has a six base pair recognition site, than for the NotI  
digest.

15 The genomes for vEL/tk and v7.5/tk were analyzed  
by Southern blotting to confirm the location of the ApaI  
and NotI sites in the HindIII J fragment as shown in Figure  
10 11. The filters were hybridized to <sup>32</sup>P labeled HindIII J  
fragment derived from the p7.5/tk or pEL/tk. The genomes  
for v7.5/tk and vEL/tk have an ApaI site that does not  
20 appear in vNotI/tk (compare lanes 7 and 8 to lane 5 in each  
blot) whereas digestion with NotI and HindIII yield a set  
of fragments of equivalent size. The 0.5 kilobase  
25 HindIII/NotI or HindIII/ApaI fragment from the left hand  
side of HindIII J produced from NotI or ApaI digestion has  
electrophoresed off the bottom of the agarose gel.

30 The definitive characterization of the promoter  
sequence utilized products of Polymerase Chain Reaction  
(PCR). A pair of primers flanking the beginning of the tk  
20 gene were used to generate a DNA fragment from the viruses  
vNotI/tk, v7.5/tk, or vEL/tk and their cognate plasmids as  
35 shown in Figure 12. The PCR products for v7.5/tk and  
vEL/tk are the same size as those observed for the plasmids  
used to generate the viruses (p7.5/tk and pEL/tk) and  
40 larger than those seen for vaccinia WR and vNotI/tk. The  
25 PCR fragments were cloned into the plasmid pCRII, the  
nucleotide sequence was determined and shown to match the  
sequence displayed in Figure 1.

#### 14.2.3. QUANTITATION OF PROMOTER ACTIVITY

The v7.5/tk and vEL/tk vectors have been designed to constitutively express elevated levels of insert protein in comparison to vNotI/tk. The level of RNA synthesis was measured by infecting confluent BSC-1 cells in the presence and absence of cytosine arabinoside (AraC) at an moi of 5, harvesting the cells, isolating the RNA using Trizol (Life Technologies) and analyzing the level of thymidine kinase RNA synthesis by primer extension (Weir, et al., 1990, Nucleic Acids Research 16:10267-10282). Incubation with AraC blocks viral DNA replication, allowing one to identify the class of viral promoter.

The early class of viral promoters are active prior to DNA replication and will be unaffected by AraC in the infection. Late promoters are only expressed after the onset of DNA replication and their activity is abrogated in the presence of AraC. Perusal of the products on a denaturing polyacrylamide gel demonstrated that significantly more (estimated to be at least ten fold) tk RNA primer extension products were synthesized in vEL/tk infections as compared to vNot/tk. In cells infected with vNot/tk a single RNA start site insensitive to AraC incubation was observed whereas in vEL/tk infections two distinct start sites, one resistant to AraC and corresponding to the appropriate early start site (Davison, et al., 1989, J. Mol. Biol. 210:749-769), and one species sensitive to AraC and corresponding to the appropriate late start of RNA (Davison, et al. 1989, J. Mol. Biol. 210:771-784) were observed (data not shown). The pattern of RNA species derived from infection with v7.5/tk was similar to that observed for vEL/tk with the absolute levels of RNA expression intermediate to that observed for vEL/tk and vNot/tk.

In order to verify the levels of expression for genes inserted into the viral vectors the *E.coli gusA* gene encoding for  $\beta$ -glucuronidase ( $\beta$ -glu) was cloned into

5 vNotI/tk, v7.5/tk and vEL/tk viral vectors and the relative  
promoter strength was measured. The DNA fragment encoding  
for the  $\beta$ -glu gene was inserted into plasmids containing  
each promoter generating pJNot/tk-GUS, p7.5/tk-GUS and  
10 pEL/tk-GUS. The correct orientation of the insert  $\beta$ -glu  
5 gene in pJNot/tk was verified by restriction enzyme  
analysis. The plasmids were recombined with vNotI/tk and  
the recombinant viruses identified by staining with X-glu  
15 (Carroll, et al., 1995, BioTechniques 19:352-355), passaged  
for three rounds through Hutk<sup>-</sup> cells, and expanded to  
generate the viral stocks vNotI/tk-GUS, v7.5/tk-GUS and  
10 vEL/tk-GUS. The structures of the recombinant viruses were  
verified by Southern blot analysis.

The level of expression of  $\beta$ -glu by vNotI/tk-GUS,  
v7.5/tk-GUS and vEL/tk-GUS was measured from infected  
confluent monolayers of BSC-1 cells in the presence or  
25 absence of AraC (Figure 13). The level of  $\beta$ -glu expression  
15 for the v7.5/tk-GUS and vEL/tk was much higher than that  
observed for vNotI/tk-GUS and highest (approximately twenty  
fold higher) in the vEL/tk-GUS. Expression of  $\beta$ -glu was  
30 observed for all three viruses in the presence of cytosine  
arabinoside, indicating that each promoter is a member of  
the early class of viral promoters. The level of  $\beta$ -glu in  
20 vNotI/tk-GUS was unchanged in the presence or absence of  
35 AraC indicating that this promoter is only active early  
during infection, whereas the  $\beta$ -glu levels in v7.5/tk-GUS  
and vEL/tk-GUS were lower in the presence of AraC,  
40 indicating these promoters are active both early and late  
times during infection.

25

#### 14.2.4. BIOCHEMICAL CHARACTERIZATION OF VIRUS VECTORS

The v7.5/tk and vEL/tk vectors were initially  
45 isolated by growth in the presence of HAT supplemented  
media and are designed to contain an active tk gene to  
allow selection for viruses with inserts via passage in  
30 Hutk<sup>-</sup> cells in the presence of bromodeoxyuridine (Earl, et

50

55

5 al., 1991, In Ausubel, et al. (eds.), Current Protocols in  
Molecular Biology. Greene Publishing Associates/Wiley  
10 Interscience, New York). Both vectors were tested by  
plaque assay in Hutk<sup>-</sup> cells using drug selection and the  
results for vEL/tk are shown in Figure 14. Incubation  
5 without drug or with HAT supplement at a concentration  
sufficient to interfere with plaque formation for vpNot or  
vNot/lacZ/tk, (data not shown), gave an equivalent number  
15 of like-sized plaques. Surprisingly, an equal number of  
plaques, albeit much smaller in size, were observed for  
vEL/tk with incubation in 25mM bromodeoxyuridine, a  
10 concentration sufficient to interfere with the ability of  
vaccinia WR to plaque on Hutk<sup>-</sup> cells (data not shown).  
Addition of 125mM bromodeoxyuridine was sufficient to  
inhibit plaque formation for vEL/tk (figure 14) and v7.5/tk  
25 (data not shown). The higher concentration of  
bromodeoxyuridine did not interfere with the growth of tk<sup>-</sup>  
15 viruses such as vNotI/lacZ/tk (data not shown) or affect  
the viability of the Hutk<sup>-</sup> cell line.

30 14.2.5. CONSTRUCTION OF RECOMBINANT  
VIRUS BY DIRECT LIGATION

20 Direct ligation vectors will only be useful for  
the generation of complex expression libraries if the  
35 production of infectious virus from the naked DNA is facile  
and efficient. Previously, helper virus activity was  
supplied in cells transfected with DNA ligation products by  
coinfection with conditionally lethal temperature sensitive  
40 virus (Merchlinsky, et al., 1992, Virology. 190:522-526) or  
25 fowlpox (Scheiflinger, et al., 1992, Proc. Natl. Acad. Sci.  
USA, 89:9977-9981). Since high levels of replicating wild  
type virus interfere with the ability to package viral DNA  
45 and vaccinia virus can recombine with the input DNA, only  
conditionally defective vaccinia virus can be used as  
30 helper (Merchlinsky, et al., 1992, Virology, 190:522-526).  
Fowlpox should be a superior helper virus as it is used at



37° C, will not revert to a highly replicating strain, and, since it does not recombine with vaccinia DNA or productively infect primate cell lines, can be used at higher moi than vaccinia. In order to determine if fowlpox can serve as an efficient helper virus a series of wells from a 12 well plate containing BSC-1 cells were infected with varying mois of fowlpox and transfected with full length vaccinia WR DNA, the cells were harvested after 24, 48, or 72 hours and the virus titer was determined as shown in Table 8. Transfection of DNA sans fowlpox or fowlpox infection alone resulted in no plaques. The level of rescued vaccinia increased with later harvest and was proportional to the moi of the fowlpox infection.

TABLE 8		
FPV moi	Day harvested	Titer (pfu x 10 <sup>-3</sup> )
0.2	1	0
	2	0.12
	3	300
0.5	1	0
	2	0.23
	3	500
1.0	1	0
	2	1.1
	3	700

Table 8. Packaging of vaccinia DNA by fowlpox virus.

Vaccinia DNA was transfected into BSC-1 cells infected with fowlpox virus using lipofectamine as described in Section 14.1 (Materials and Methods). The cells were harvested at 1, 2, or 3 days post transfection, lysed by freeze-thaw cycles and assayed for infectious virus by plaque assay on BSC-1 cells.

A 1.1 kilobase pair fragment of the ovalbumin cDNA (Pulaski, et al., 1996, Proc. Natl. Acad. Sci. USA

93:3669-3674) was used as a model insert to study the generation of functional recombinant virus by direct ligation. The ovalbumin insert was modified as described in the Materials and Methods to include a NotI site at its 5' end, translation stop codons, a vaccinia transcription stop signal and an ApaI site at its 3' end. This insert was digested with NotI and ApaI and ligated with purified vEL/tk DNA arms that had been digested with NotI and ApaI. The ligation mix was transfected into fowlpox infected BSC-1 cells, cells were harvested, and after three days the cell extract was passaged on Hutk<sup>-</sup> cells in the presence or absence of 125mM bromodeoxyuridine. The titer obtained without drug selection was  $2.7 \times 10^5$  pfu and with drug selection  $2.8 \times 10^5$  pfu. Individual plaques were picked from Hutk<sup>-</sup> cells in the presence and absence of bromodeoxyuridine and tested for the presence of the ovalbumin insert by dot blot hybridization with an ovalbumin cDNA probe. All 15 plaques picked in the presence of bromodeoxyuridine, and all 10 plaques picked in its absence contained the ovalbumin insert. These viruses were named vEL/tk-ovaFL. Two individual clones were expanded further and tested for the ability to sensitize host cells to lysis by ova 257-264 specific cytotoxic T lymphocytes (CTL). The results of this experiment are shown in Table 9. As controls, vaccinia recombinant for an ova 257-264 minigene, v7.5/tk-ova and vEL/tk- ova, were generated by homologous recombination. These ova peptide recombinant viruses were tested in concert with the vEL/tk-ovaFL clones for the ability to sensitize host cells to lysis by ova specific CTL. As shown in Table 9, infection with either full length or minigene ovalbumin vaccinia recombinants was as efficient as pulsing with  $1 \mu\text{M}$  purified OVA 257-264 peptide for sensitization of target cells to lysis by OVA-specific CTL.

TABLE 9

MC57G cells:	Effector:Target Ratio	
	2:1	10:1
	(Percent Specific Lysis)	
Untreated	-1.3	-1.3
ova257-264 peptide, 1 $\mu$ M	54	83
vEL/tk	-0.5	0
v7.5/tk-ova Homologous Recombination	50	78
vEL/tk-ova Homologous Recombination	47	71
vEL/tk-ovaFL Direct Ligation Clone 1	48	70
vEL/tk-ovaFL Direct Ligation Clone 2	46	74

Table 9. CML assay on recombinant vaccinia virus infected cells. Virally infected MC57G cells were generated as described in Section 14.1 (Materials and Methods). One sample of MC57G cells was treated with ova257-264 peptide (1 $\mu$ M), another sample of cells was left untreated. Cells were incubated with two different ratios of ova specific cytotoxic T lymphocytes for 4 hours at 37° C and percent specific lysis was determined as described in Section 14.1 (Materials and Methods).

#### 14.3. DISCUSSION

Large DNA viruses are particularly useful expression vectors for the study of cellular processes as they can express many different proteins in their native form in a variety of cell lines. In addition, gene products expressed in recombinant vaccinia virus have been shown to be efficiently processed and presented in association with MHC class I for stimulation of cytotoxic T cells. The gene of interest is normally cloned in a plasmid under the control of a promoter flanked by sequences homologous to a non-essential region in the virus and the cassette is introduced into the genome via

5 homologous recombination. A panoply of vectors for  
expression, selection and detection have been devised to  
accommodate a variety of cloning and expression strategies.  
10 However, homologous recombination is an ineffective means  
of making a recombinant virus in situations requiring the  
5 generation of complex libraries or when the insert DNA is  
large. An alternative strategy for the construction of  
recombinant genomes relying on direct ligation of viral DNA  
15 "arms" to an insert and the subsequent rescue of infectious  
virus has been explored for the genomes of poxvirus  
(Merchlinisky, et al., 1992, Virology 190:522-526;  
10 Pfleiderer, et al., 1995, J. General Virology 76:2957-2962;  
Scheifflinger, et al., 1992, Proc. Natl. Acad. Sci. USA  
20 89:9977-9981), herpesvirus (Rixon, et al., 1990, J. General  
Virology 71:2931-2939) and baculovirus (Ernst, et al.,  
1994, Nucleic Acids Research 22:2855-2856).

25 Poxviruses are ubiquitous vectors for studies in  
15 eukaryotic cells as they are easily constructed and  
engineered to express foreign proteins at high levels. The  
wide host range of the virus allows one to faithfully  
30 express proteins in a variety of cell types. Direct  
cloning strategies have been devised to extend the scope of  
applications for poxvirus viral chimeras in which the  
20 recombinant genomes are constructed in vitro by direct  
ligation of DNA fragments to vaccinia "arms" and  
35 transfection of the DNA mixture into cells infected with a  
helper virus (Merchlinisky, et al., 1992, Virology 190:522-  
526; Scheifflinger, et al., 1992, Proc. Natl. Acad. Sci. USA  
40 89:9977-9981). This approach has been used for high level  
25 expression of foreign proteins (Pfleiderer, et al., 1995,  
J. Gen. Virology 76:2957-2962) and to efficiently clone  
fragments as large as 26 kilobases in length (Merchlinisky,  
45 et al., 1992, Virology 190:522-526).

Vaccinia virus DNA is not infectious as the virus  
cannot utilize cellular transcriptional machinery and  
30 relies on its own proteins for the synthesis of viral RNA.

5 Previously, temperature sensitive conditional lethal  
(Merchlinisky, et al., 1992, Virology 190:522-526) or non-  
homologous poxvirus fowlpox (Scheifflinger, et al., 1992,  
10 Proc. Natl. Acad. Sci. USA 89:9977-9981) have been utilized  
as helper virus for packaging. An ideal helper virus will  
5 efficiently generate infectious virus but not replicate in  
the host cell or recombine with the vaccinia DNA products.  
15 Fowlpox virus has the properties of an ideal helper virus  
as it is used at 37° C, will not revert to a highly  
replicating strain, and, since it does not recombine with  
vaccinia DNA or productively infect primate cell lines, can  
10 be used at relatively high moi.

The utility of the vaccinia based direct ligation  
vector vNotI/tk, has been described by Merchlinisky, et al.  
(1992, Virology 190:522-526). This genome lacks the NotI  
25 site normally present in the HindIII F fragment and  
contains a unique NotI site at the beginning of the  
15 thymidine kinase gene in frame with the coding sequence.  
This allows the insertion of DNA fragments into the NotI  
site and the identification of recombinant genomes by drug  
30 selection. The vNotI/tk vector can be used to efficiently  
clone large DNA fragments but does not fix the orientation  
of the DNA insert or lead to high expression of the foreign  
20 protein. This example describes the construction and  
characterization of a pair of vaccinia DNA vector genomes  
35 v7.5/tk and vEL/tk suitable for direct ligation. The  
v7.5/tk and vEL/tk vectors were designed to contain unique  
restriction sites for NotI and ApaI at the beginning of the  
40 thymidine kinase gene allowing the oriented cloning of DNA  
25 and eliminating the intact genomes arising from relegation  
of vaccinia vector arms.

The vNotI/tk vector will only express foreign  
45 proteins at the level of the thymidine kinase gene, a  
weakly expressed gene only made early during viral  
infection. To induce high levels of protein expression the  
30 sequences encoding for the viral 7.5k promoter and a  
50 synthetic EL promoter devised by Chakrabarti and Moss were

5 used to replace the endogenous thymidine kinase promoter.  
The levels of expression induced by either promoter was  
much higher than that observed in vNotI/tk and the  
10 promoters were active at all times post infection. These  
continuous expression vectors are applicable in cases  
5 dependent on early expression, such as T-cell epitope  
presentation, as well as for bulk expression of proteins.  
Use of the thymidine kinase gene as the insertion  
15 site for foreign DNA allows implementation of selection  
protocols for distinguishing recombinants from helper or  
wild type genomes. The level of tk expression in v7.5/tk  
10 and vEL/tk should be much higher than in vaccinia WR or  
vNot/tk. However, the ApaI site at the beginning of the tk  
20 gene in v7.5/tk and vEL/tk was formed from vNot/tk by  
adding extra nucleotides at the NotI site. The additional  
nucleotides increase the amino acid sequence at the N  
25 terminus of the wild type tk gene from Met-Asn-Gly to Met-  
15 Gly-Pro-Ala-Ala-Asn-Gly in v7.5/tk and vEL/tk.  
Modifications in the expression level and N terminal amino  
acid sequence of the thymidine kinase gene may increase  
30 (more protein) or decrease (different sequence) the  
sensitivity of the virus to bromodeoxyuridine. Plaques,  
albeit smaller, were observed with v7.5/tk and vEL/tk  
20 infection at a concentration of bromodeoxyuridine  
sufficient to completely suppress plaque formation for wild  
35 type vaccinia WR. Plaque formation was suppressed at five-  
fold higher concentrations of bromodeoxyuridine, a level of  
drug that does not interfere with the viability of the  
40 cells or impede the ability of tk<sup>-</sup> virus to form plaques.  
25 The explanation for the altered sensitivity to  
bromodeoxyuridine awaits further characterization of the  
protein as the altered thymidine kinase gene may have a  
45 different reaction rate for formation of the triphosphate  
form of the bromodeoxyuridine or a reduced ability to bind  
bromodeoxyuridine.  
30 The development of direct ligation vectors has  
increased the possible applications for poxvirus expression  
50

5 vectors. The v7.5/tk and vEL/tk vectors were designed to  
incorporate the advantages of oriented cloning, high levels  
of expression of foreign protein, and the selection for  
10 recombinant viruses, into direct ligation vectors. They  
were shown to express high levels of proteins at all times  
5 during infection. The utility of these vectors was  
demonstrated by constructing recombinants containing a CTL  
epitope for ovalbumin (constructed by homologous  
15 recombination with a plasmid) or the ovalbumin coding  
sequence (constructed by direct ligation protocol) and  
showing how both recombinants were able to elicit a strong  
10 CTL response

20 The application of these vectors to protocols for  
construction of complex expression libraries requires  
efficient production of recombinants and strong selection  
to eliminate or minimize wild type and contaminants. The  
25 use of two restriction sites allows one to design cloning  
15 strategies for the oriented cloning of DNA fragments such  
as products of PCR (Pfleiderer, et al., 1995, J. General  
Virology 76:2957-2962) and increases the frequency of the  
desired recombinant as wild type genomes can no longer be  
30 generated by ligation of vaccinia arms. When v7.5/tk or  
vEL/tk DNA previously digested with NotI and ApaI was  
20 transfected into cells infected with fowlpox the virus  
titer was one hundred fold lower than for intact uncut DNA.  
35 Also, all plaques isolated in the presence and absence of  
bromodeoxyuridine (15 with bromodeoxyuridine and 10  
without) during the isolation of the vEL/tk-ovaFL contained  
40 the ovalbumin insert. The efficiency of infectious virus  
25 formation is also increased with the use of fowlpox, helper  
virus at relatively high moi. Also, transfection of large  
DNA fragments varies with the type and preparation of lipid  
45 (Miles Carroll, personal communication) and we are  
presently assaying different lipid mixtures and cell types  
as well as investigating other parameters to find optimum  
30 conditions for the direct ligation protocol. The v7.5/tk

5 and vEL/tk vectors provide a set of universally applicable  
direct ligation cloning vectors for poxviruses.

10 The present invention is not to be limited in  
scope by the specific embodiments described which are  
5 intended as single illustrations of individual aspects of  
the invention, and any constructs, viruses or enzymes which  
are functionally equivalent are within the scope of this  
15 invention. Indeed, various modifications of the invention  
in addition to those shown and described herein will become  
apparent to those skilled in the art from the foregoing  
20 description and accompanying drawings. Such modifications  
are intended to fall within the scope of the appended  
claims.

All publications and patent applications  
mentioned in this specification are herein incorporated by  
25 reference to the same extent as if each individual  
15 publication or patent application was specifically and  
individually indicated to be incorporated by reference.



## Claims

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WHAT IS CLAIMED IS:

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1. A method for identifying a target epitope comprising screening products of an expression library generated from DNA or RNA derived from a cell expressing the target epitope with cytotoxic T cells generated against the cell to identify DNA clones expressing the target epitope.

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2. The method of Claim 1 wherein the target epitope is specific to a cell infected with a virus, fungus or mycobacteria.

10

20

3. The method of Claim 1 wherein the target epitope is specific to an autoimmune disease.

25

4. The method of Claim 1 wherein the expression library is constructed in a viral vector infectious for mammalian cells.

30

5. The method of Claim 4 wherein the viral vector is constructed by trimolecular recombination.

35

6. The method of Claim 4 wherein the viral vector is a vaccinia viral vector.

40

7. A method for identifying a tumor specific target epitope comprising screening products of an expression library generated from DNA or RNA derived from a tumor cell expressing the target epitope with cytotoxic T cells generated against the tumor cell to identify DNA clones expressing the target epitope.

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8. The method of Claim 7 wherein the cytotoxic T cells react with tumor cells derived from a non-tumorigenic cell line and do not cross-react with the non-tumorigenic cell line.

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10 9. The method of Claim 7 wherein the cytotoxic T cells are derived from animals tolerized with a non-tumorigenic cell line and are then immunized with tumor cells derived from the non-tumorigenic cell line.

15 10. The method of Claim 9 wherein the cytotoxic T cells are derived from animals tolerized with a non-tumorigenic cell line that does not express costimulator activity and are subsequently stimulated with a tumor cell line expressing costimulator activity.

20 11. The method of Claim 7 wherein the genes expressed in tumor cells are used to generate HLA restricted cytotoxic T cells which are evaluated for activity against tumor cells.

25 12. The method of Claim 7 wherein the tumor cell is  
15 derived from a single immortalized, non-tumorigenic cell line.

30 13. The method of Claim 12 wherein the screening is performed on a panel of tumor cell lines each derived independently from a single non-tumorigenic cell.  
20

35 14. The method of Claim 7 wherein the expression library is constructed in a viral vector infectious for mammalian cells.

40 15. The method of Claim 14 wherein the viral vector  
25 is constructed by trimolecular recombination.

45 16. The method of Claim 14 wherein the viral vector is a vaccinia viral vector.

50 17. A method for identifying a target epitope or  
30 antigen comprising:

5 (a) providing cytotoxic T cells specific for a  
gene product differentially expressed by a cell expressing  
the target epitope, and

10 (b) measuring crossreactivity of the cytotoxic T  
cells for the cell in which target epitopes are identified  
5 as the gene product which induces cytotoxic T cells.

15 18. The method of Claim 17 wherein the target epitope  
is specific to a cell infected with a virus, fungus or  
mycobacteria.

20 19. The method of Claim 17 wherein the target epitope  
is specific to an autoimmune disease.

25 20. The method of Claim 17 wherein a modified  
differential display method is employed that increases  
resolution of DNA fragments and reduces the frequency of  
15 false positives.

30 21. The method of Claim 20 further comprising use of  
the DNA fragments to isolate longer gene products following  
solution hybridization to single strand circles rescued  
from a phagemid DNA library.

35 22. A method for identifying a tumor specific target  
epitope or antigen comprising:

40 (a) providing cytotoxic T cells specific for a  
gene product differentially expressed by a tumor cell  
expressing the target epitope, and

25 (b) measuring crossreactivity of the cytotoxic T  
cells for the tumor cell in which target epitopes are  
identified as the gene product which induces cytotoxic T  
45 cells.

50 23. The method of Claim 22 wherein the tumor cell is  
derived from a single immortalized, non-tumorigenic cell  
line.

5

24. The method of Claim 22 wherein the assay is performed on a panel of tumor cell lines each derived independently from a single non-tumorigenic cell.

10

25. The method of Claim 22 wherein the generated cytotoxic T cells which react to tumor cells do not react to nontumorigenic T cells.

15

26. The method of Claim 22 wherein a modified differential display method is employed that increases resolution of DNA fragments and reduces the frequency of false positives.

20

27. The method of Claim 26 further comprising use of the DNA fragments to isolate longer gene products following solution hybridization to single strand circles rescued from a phagemid DNA library.

25

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28. A viral vector containing a DNA insert flanked by unique sites for restriction enzymes positioned so that religation of the viral vectors arms is prevented and the orientation of the insert DNA is fixed and the DNA insert is operatively associated with a strong regulatory element.

30

20

29. The viral vector of Claim 28 wherein the vector is constructed by trimolecular recombination.

35

30. The viral vector of Claim 28 wherein the viral vector is a vaccinia viral vector.

40

25

31. The vector of Claim 28 in which the vector is derived by recombination with plasmid p7.5/tk (SEQ ID NO: ) or derivatives thereof.

45

32. The vector of Claim 28 in which the vector is derived by recombination with plasmid pEL/tk (SEQ ID NO: ) or derivatives thereof.

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33. A viral vector containing a DNA insert flanked by unique sites for restriction enzymes positioned so that religation of the viral vectors arms is prevented and the orientation of the insert DNA is fixed and the DNA insert is operatively associated with a strong regulatory element wherein the DNA insert encodes a target epitope identified by the method of Claim 1.

10

15

34. A viral vector containing a DNA insert flanked by unique sites for restriction enzymes positioned so that religation of the viral vectors arms is prevented and the orientation of the insert DNA is fixed and the DNA insert is operatively associated with a strong regulatory element wherein the DNA insert encodes a target epitope identified by the method of Claim 7.

20

25

35. The viral vector of Claim 33 or 34 wherein the viral vector is a vaccinia viral vector.

30

36. A vaccinia viral vector containing a DNA insert flanked by unique sites for restriction enzymes positioned so that religation of the viral vectors arms is prevented and the orientation of the insert DNA is fixed and the DNA insert is operatively associated with a strong regulatory element wherein the DNA insert encodes a target epitope identified by the method of Claim 17.

35

40

37. A vaccinia viral vector containing a DNA insert flanked by unique sites for restriction enzymes positioned so that religation of the viral vectors arms is prevented and the orientation of the insert DNA is fixed and the DNA insert is operatively associated with a strong regulatory element wherein the DNA insert encodes a target epitope identified by the method of Claim 22.

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38. The viral vector of Claim 36 or 37 wherein the viral vector is a vaccinia viral vector.

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39. A transgenic animal tolerized with a non-tumorigenic cell line that does not express costimulator activity.

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15

40. A transgenic animal according to claim 39, wherein said transgenic animal is further stimulated with a tumor cell line expressing costimulator activity.

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41. A cytotoxic T cell derived from the transgenic animal of claim 39.

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42. A cytotoxic T cell derived from the transgenic animal of claim 40.

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SUBSTITUTE SHEET (RULE 26)

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1. p7.5tk			
	7.5K PROMOTER	NOTI	APAI
5'-GGCCAAAAATTGAAAAAAGTAGATCTATTATTGACGCGGGCCGCGCATGGGCCCGGCC-3'			
2. p7.5/ATG0/tk			
	7.5K PROMOTER	NOTI	BAMHI SMAI PSTI
5'-GGCCAAAAATTGAAAAAAGTAGATCTATTATTGACGCGGGCCGCGCGATCCCCGGGCTGCAGGAA			
	SALI	TRANSLATION	TRANSCRIPTION
		STOP CODONS	STOP SIGNAL
TTCGATATCAAGCATTATCGATACCGTCGACCTCGAGGGGGGCGCTAACTAAATTGTTTTTGT			
APAI			
GGGCCCGGCC-3'			

FIG. 2

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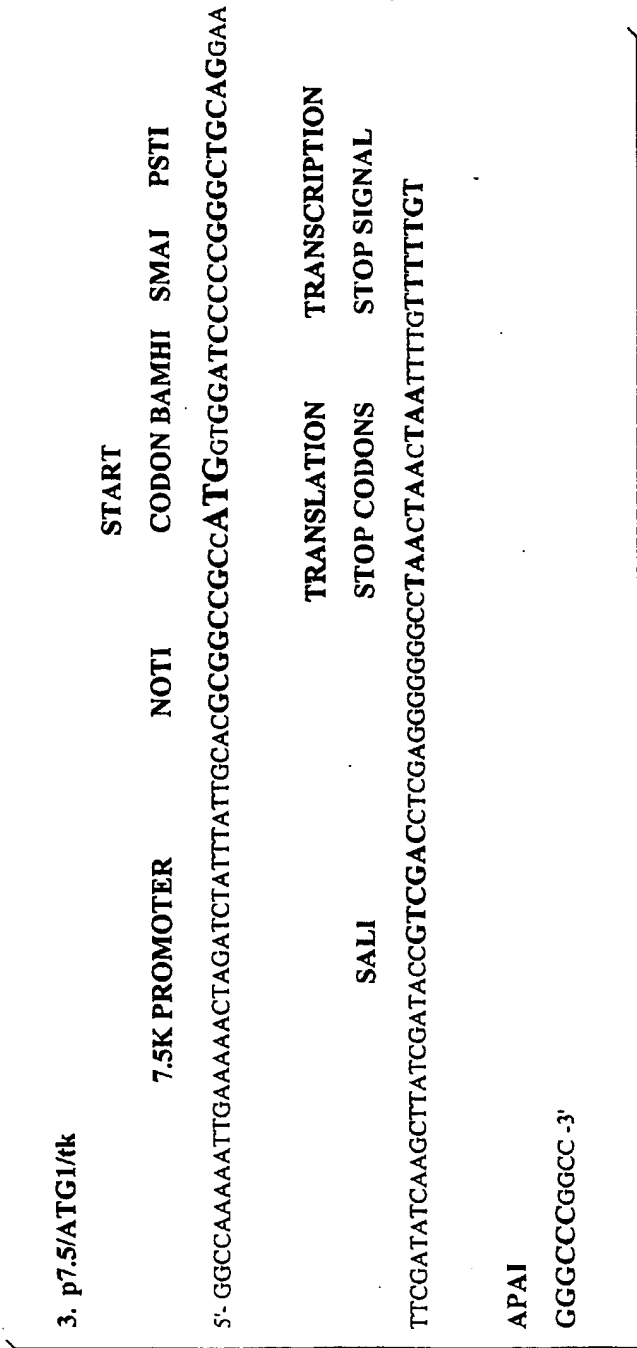


FIG. 2 (cont.)

4. p7.5/ATG2/tk

7.5K PROMOTER NOTI CODON BAMHI SMAI PSTI START

5'- GGCCAAAAATTGAAAAAAGTAGATCTATTATTGACGGGGGGCCCAATGAGTGGATCCCCCGGGCTGCAGGAA

SALI TRANSLATION TRANSCRIPTION

STOP CODONS STOP SIGNAL

TTCGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCTAACTAAATTTGTTTGT

APAI

GGGCCCCGGCC -3'

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*FIG. 2 (cont.)*

5. p7.5/ATG3/tk	START				
	7.5K PROMOTER	NOTI	CODON	BAMHI SMAI	PSTI
5'- GGCCAAAATTGAAAACTAGATCTATTTATTGCACGGGGCCGccATGACGTGGATCCCCGGGCTGCAGGAA					
			TRANSLATION	TRANSCRIPTION	
	SALI		STOP CODONS	STOP SIGNAL	
TTCGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGCCCTAACTAATTGTTTTTGT					
APAI					
GGGCCCGGCC -3'					

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**FIG. 2 (cont.)**

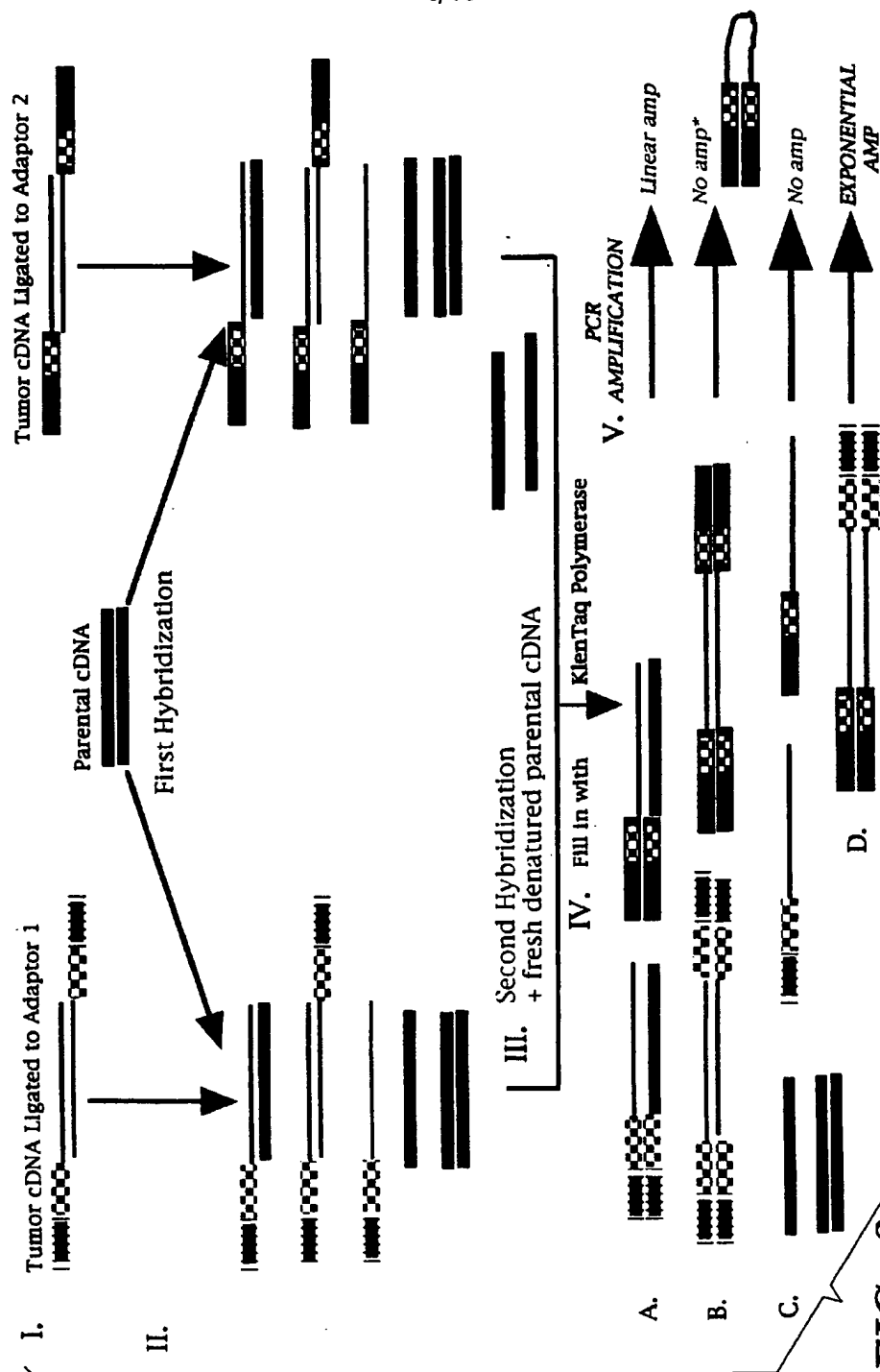
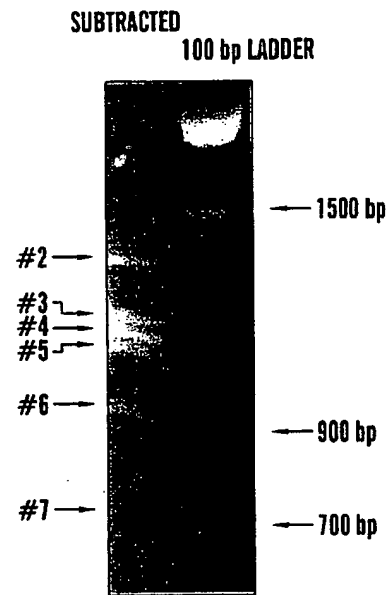


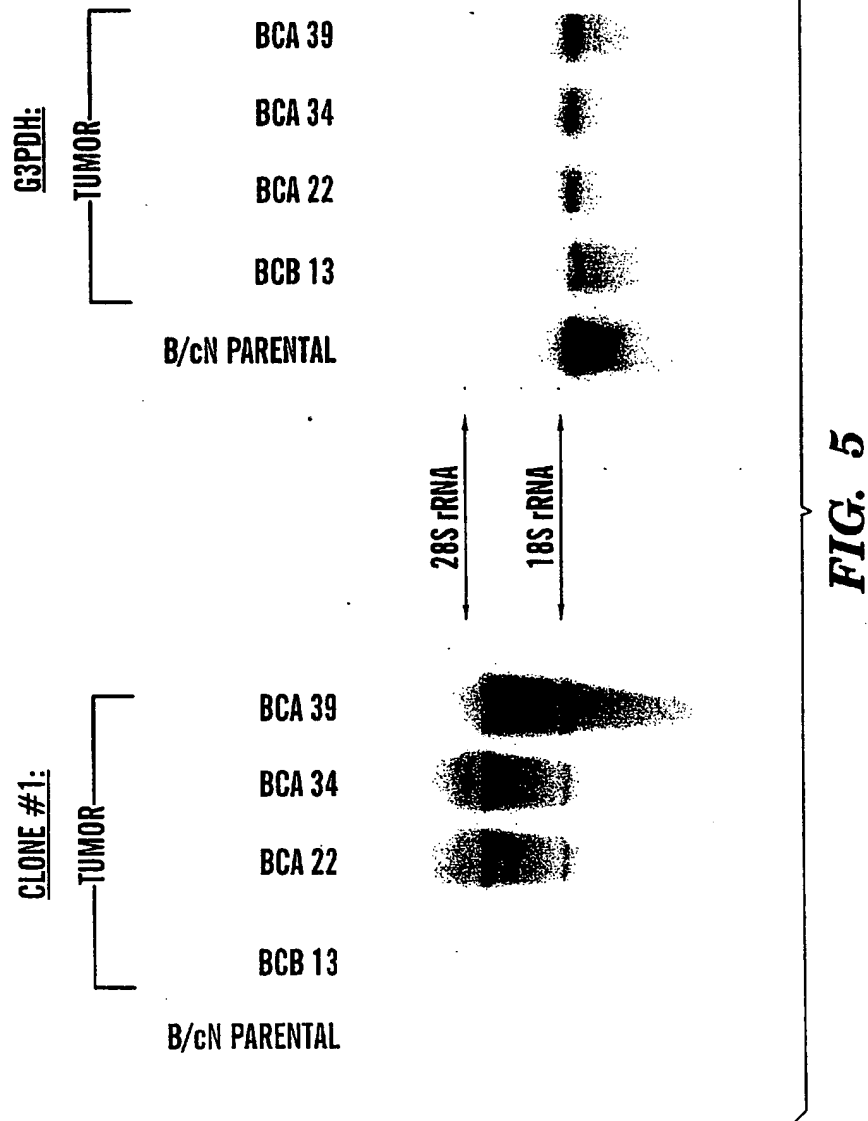
FIG. 3

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**FIG. 4**

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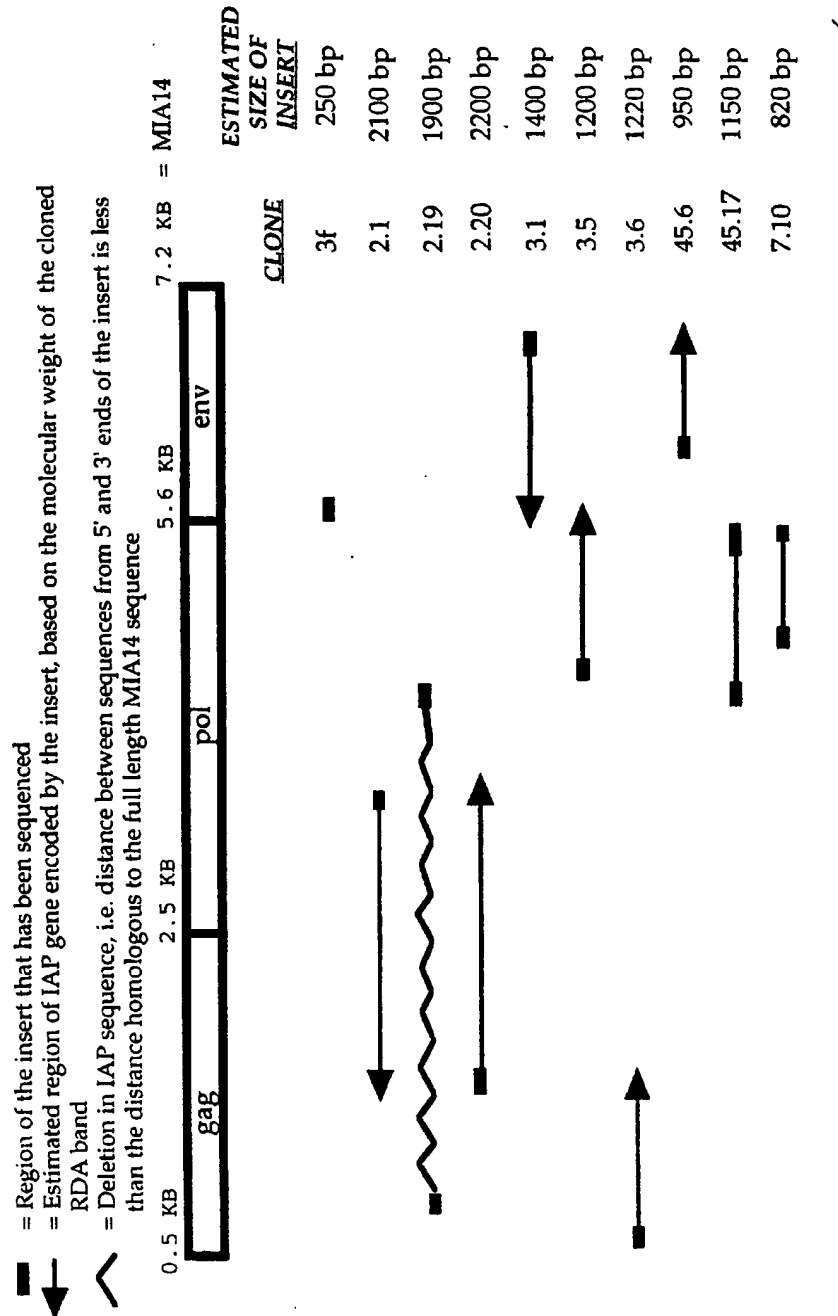
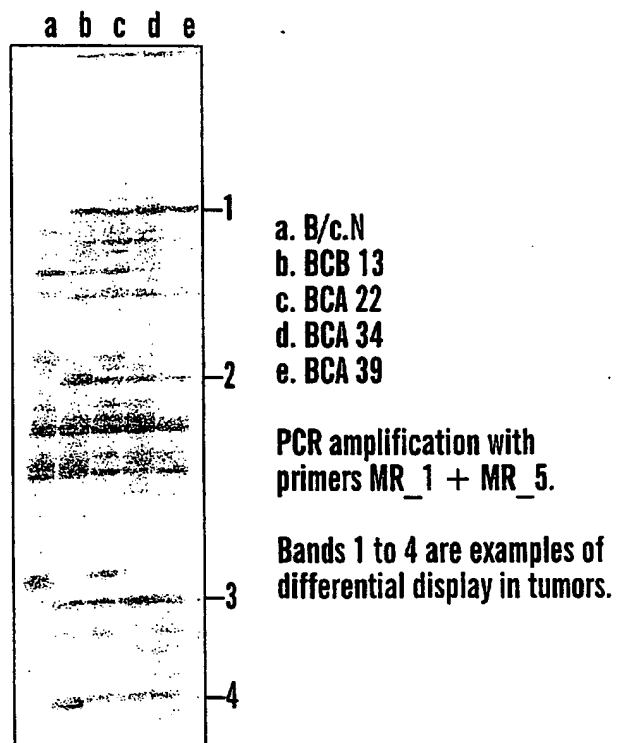


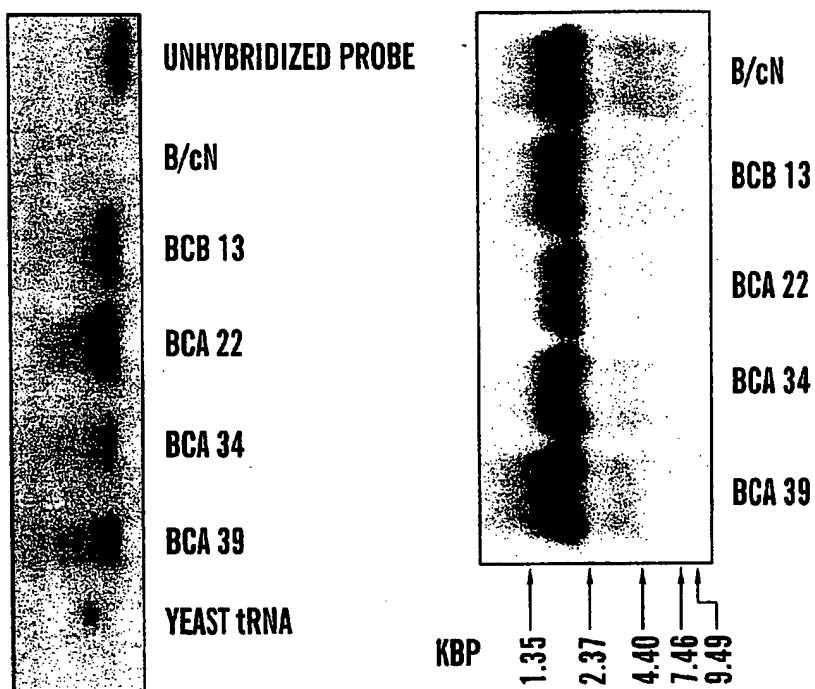
FIG. 6



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**FIG. 7**

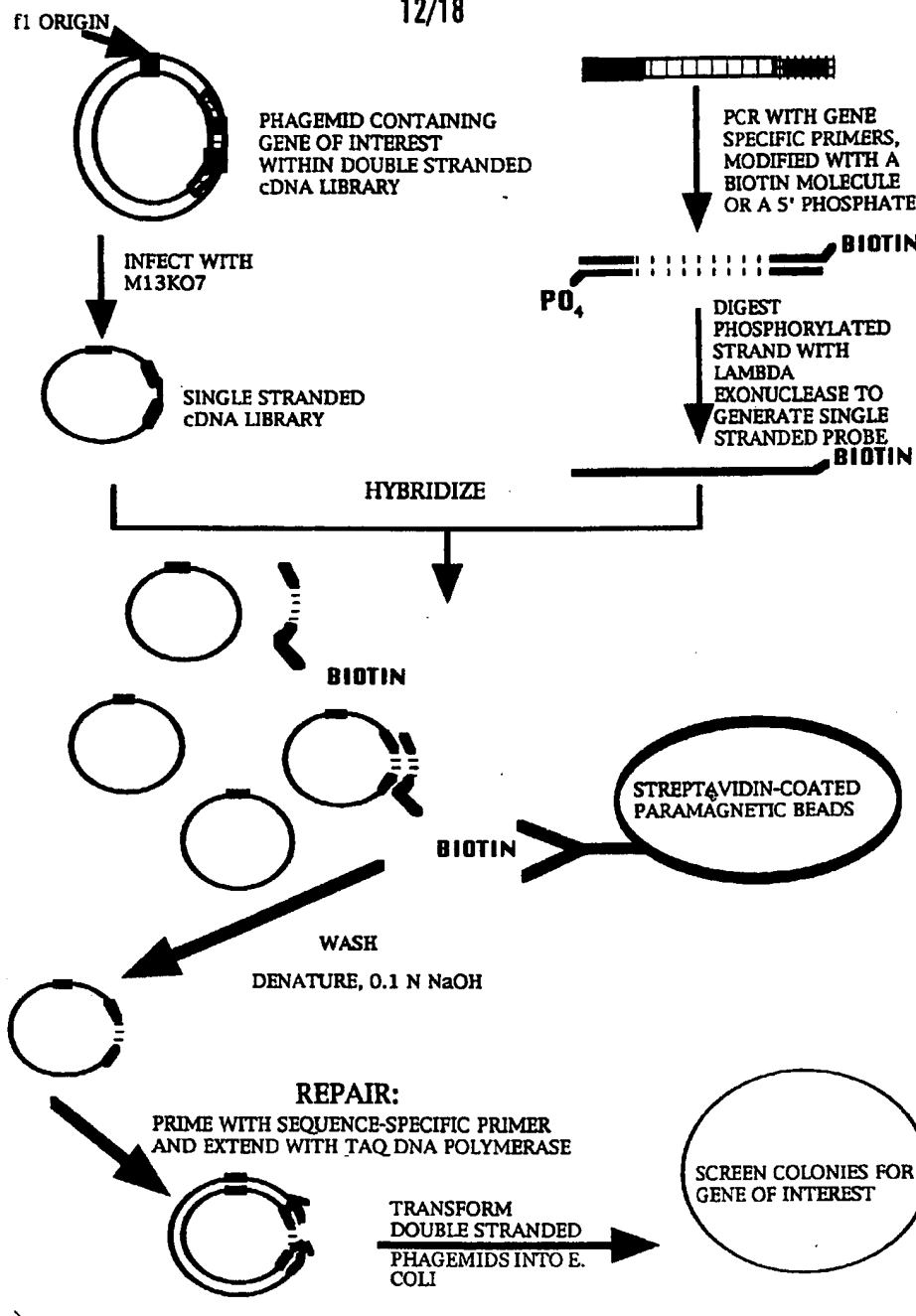
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**FIG. 8A**

**FIG. 8B**

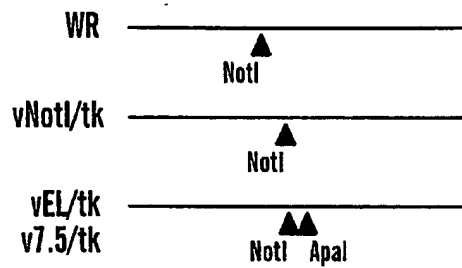
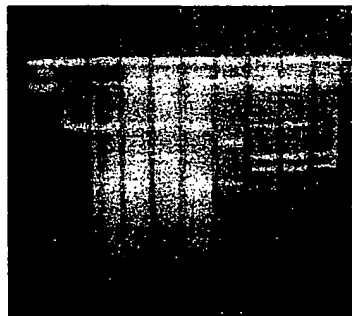
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**FIG. 9**

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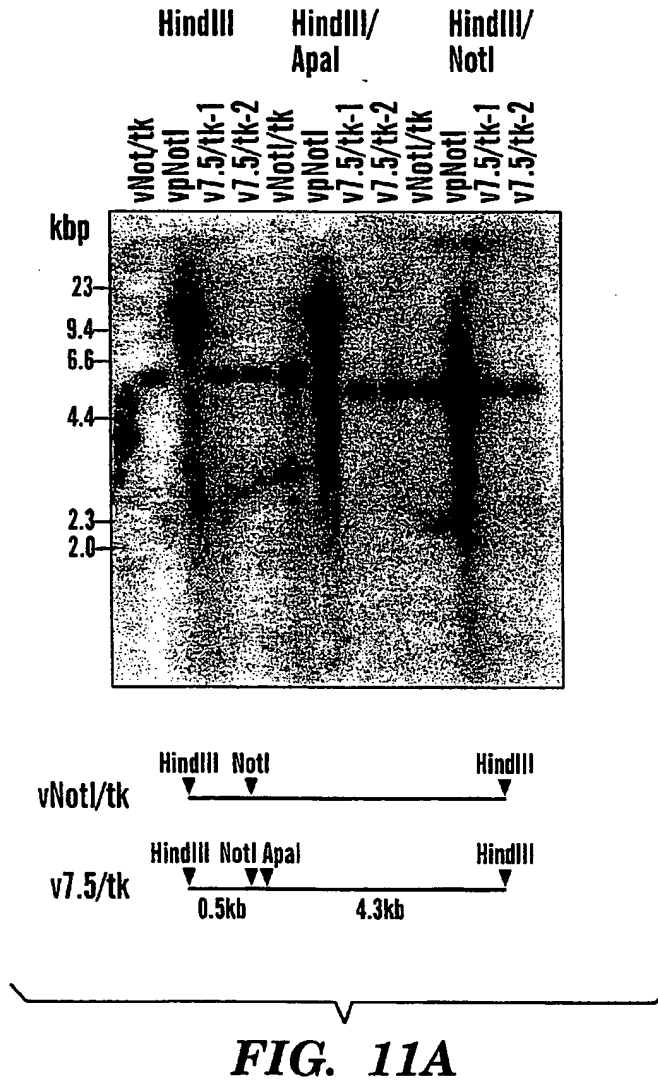
Apal Notl

$\lambda$  WR WR vEL v7.5 vNot/tk  
WR WR vEL v7.5 vNot/tk

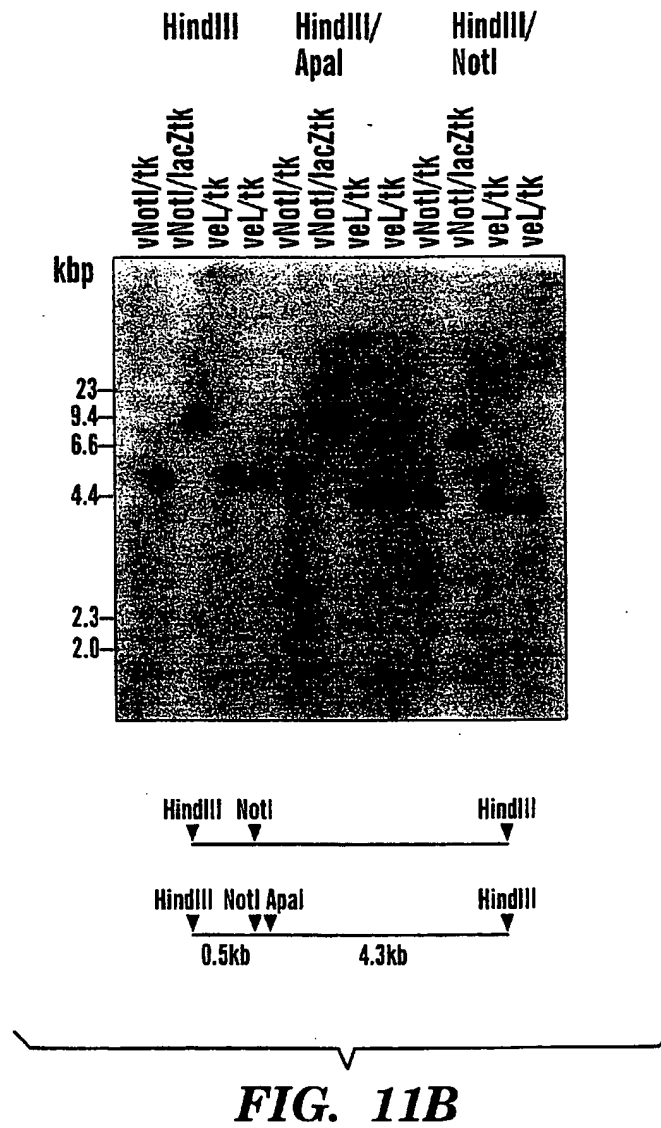


**FIG. 10**

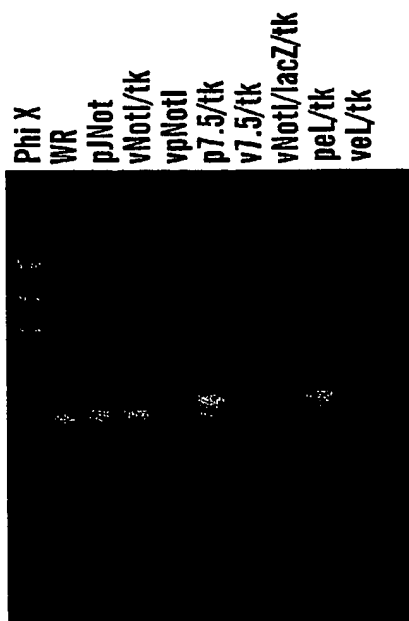
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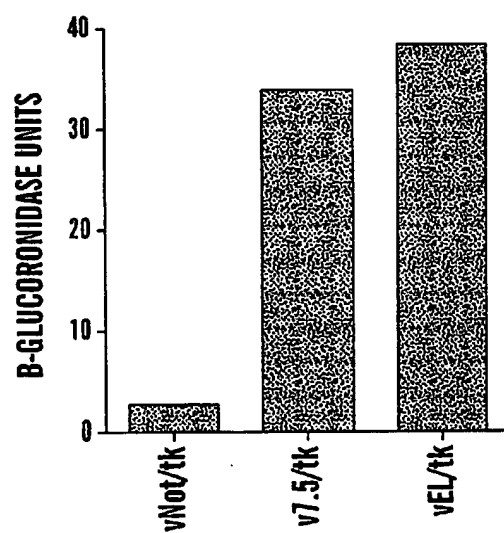


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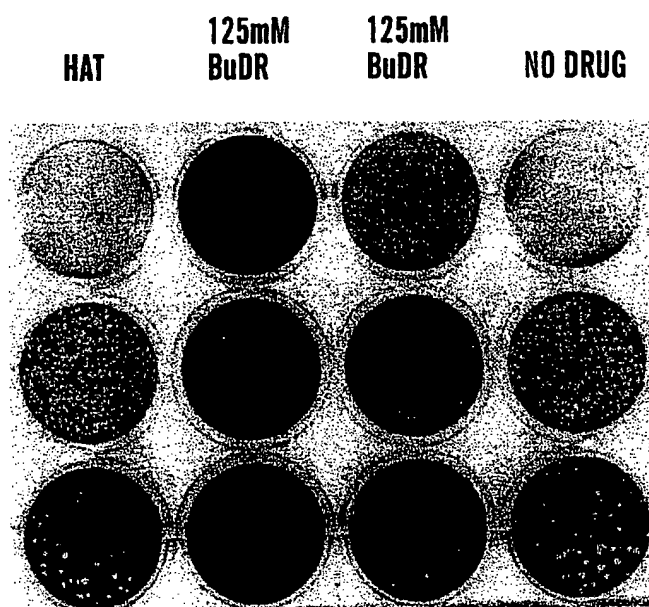
**FIG. 12**

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**FIG. 13**



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**FIG. 14**

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 98/24029

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/10 C12N15/86 C12N5/06 C12Q1/68 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N C12Q A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 34143 A (UNIV BRITISH COLUMBIA :JEFFERIES WILFRED A (CA); GABATHULER REINHA) 18 September 1997	1-4,6,7, 11,14, 16-19, 22,25
Y	see claims 1-11	5,15
X	R.-F. WANG ET AL.: "Development of a retrovirus-based complementary DNA expression system for the cloning of tumor antigens" CANCER RESEARCH, vol. 58, 15 August 1998, pages 3519-3525, XP002107625 AACR, BALTIMORE, US see the whole document	1,4,7,8, 14,17, 22,25

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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search

29 June 1999

Date of mailing of the international search report

12/07/1999

Name and mailing address of the ISA  
European Patent Office, P.B. 5818 Patentlaan 2  
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Authorized officer

Hornig, H

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 98/24029

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M. MERCHLINSKY ET AL.: "Construction and characterization of vaccinia direct ligation vectors" VIROLOGY, vol. 238, no. 2, 24 November 1997, pages 444-451, XP002107626 ACADEMIC PRESS, INC., NEW YORK, US see the whole document	28-38
Y	see the whole document	5, 15
A	WO 97 26328 A (RPMS TECHNOLOGY LTD ;STAUSS HANS JOSEF (GB)) 24 July 1997 see the whole document	1-42
A	WO 95 33835 A (CHIRON CORP ;SELBY MARK (US); WALKER CHRISTOPHER (US)) 14 December 1995 see the whole document	1-42
A	WO 97 24438 A (ACTIVATED CELL THERAPY INC ;LAUS REINER (US); RUEGG CURTIS LANDON) 10 July 1997 see the whole document	1-42
A	TAKAHASHI H ET AL: "INDUCTION OF CD8+ CYTOTOXIC T CELLS BY IMMUNIZATION WITH PURIFIED HIV-1 ENVELOPE PROTEIN IN ISCOMS" NATURE, vol. 344, 26 April 1990, pages 873-875, XP002031410 see the whole document	1-42
A	"Clonetechn PCR-Select™ Subtraction" CLONETECH CATALOG 98/99, 1998, page 24 XP002107627 Clonetechn laboratories, Palo Alto, Ca, US cited in the application see figure 2.4	1-42
A	US 5 804 382 A (YANG MEIHENG ET AL) 8 September 1998 see the whole document	1-42
A	D.M. SAHASRABUDHE ET AL.: "Shared T cell-defined antigens on independently derived tumors" J. OF IMMUNOLOGY, vol. 151, no. 11, 1 December 1993, pages 6302-6310, XP002107628 WAVERLY PRESS, BALTIMORE, MD, US cited in the application see the whole document	1-42

1

Form PCT/ISA210 (continuation of second sheet) (July 1992)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/24029

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-27,39-42

A method for identifying a target epitope comprising screening products from an expression library generated from DNA or RNA derived from cells expressing the target epitope with cytotoxic T cells generated against the cell to identify DNA clones expressing the target epitope; a transgenic animal tolerized with a non-tumorigenic cell line that does not express costimulatory activity; a cytotoxic T cell derived from said transgenic animal;

2. Claims: 28-38

A viral vector containing a DNA insert flanked by unique sites for restriction enzymes positioned so that religation of the viral vector arms is prevented and the orientation of the insert DNA is fixed and the DNA insert is operatively associated with a strong regulatory element.

# INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 98/24029

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